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Internship at the sole hatchery Safiestela, SA. Assessment of the *Pediococcus acidilactici* probiotic effects on growth performance of *Solea senegalensis.*

Dissertation presented according to the requirements for the degree in Master in Marine Sciences - Marine Resources, Specialization in Aquaculture and Fisheries, submitted to Institute of Biomedical Sciences Abel Salazar, University of Porto.

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Abstract

Aquaculture is an important economic activity in several countries. In Portugal the production of aquatic organisms is around 9000 tons and the most important marine fish species produced are seabass, seabream, turbot and sole. The Senegalese sole is a flat fish with a high commercial value that draws the attention of investors to Portugal. The company “Safiestela, SA” is an intensive unit of sole production in the north of Portugal.

The intensive production of *Solea senegalensis* exposes these animals to different stressor agents that may weaken their immune system and diminish their growth and, consequently, inducing the occurrence of diseases and environmental degradation. The emerging concern for human health and the environment led to the use of probiotics in fish, allowing to prevention and control of diseases and also promoting higher growth and weight gain, as an alternative to antibiotics.

The Bactocell probiotic is a feed additive based on viable cells of a lactic acid bacteria *Pediococcus acidilactici* (PA). In the present work, a brief Bactocell PA administration during the larval phase was added in two different trials to evaluate the influence of this bacterial strain in the sole growth performance and its capacity to colonize the larvae intestine. It was concluded that the larvae growth of the groups fed with probiotics was higher than the control group, with a statically significance in the 7, 9 and 31 days after hatching (DAH). The administration of this probiotic diminished the sole growth heterogeneity, with statistically significant differences ($p<0.05$) in 20 and 45 DAH. These results demonstrated that, with this probiotic administration, is possible to diminish the handling during the size grading and competitive behavior of the soles, decreasing the number of stress situations and, consequently, increasing growth performance. In relation to the gut microbiota it was demonstrated that the two feeding regimes promote the dominance of different bacterial populations in the two treatment groups, which suggests that Bactocell has the capability to modulate the gut microbiota of sole larvae.

Keywords: *Solea senegalensis*, sole, probiotic, Bactocell

Resumo

A aquacultura é uma atividade económica importante em diferentes países. Em Portugal, a produção de organismos aquáticos ronda as 9000 toneladas e as espécies mais importantes de peixes marinhos produzidos são o robalo, a dourada, o pregado e o linguado. O linguado é um peixe chato com um elevado valor comercial que chamou a atenção de investidores para Portugal. A empresa "Safiestela, SA" é uma unidade intensiva de produção de linguado no norte de Portugal.

A produção intensiva de *Solea senegalensis* envolve a presença de diferentes agentes de stress que podem enfraquecer o seu sistema imunológico e diminuir o seu crescimento. Além disso, a preocupação emergente para a saúde humana e para o ambiente levaram à utilização de probióticos que permitem a prevenção e controlo de doenças e também promovem um maior crescimento e ganho de peso, como uma alternativa aos antibióticos.

O probiótico Bactocell é um suplemento alimentar com base em células viáveis de bactérias do ácido láctico *Pediococcus acidilactici* (PA). No presente trabalho, Bactocell PA foi administrado durante um pequeno período de tempo durante a fase larval em dois ensaios para avaliar a influência desta estirpe de bactérias na performance de crescimento do linguado e a sua capacidade de colonizar o intestino das larvas. Concluiu-se que o crescimento das larvas dos grupos alimentados com probióticos foi maior do que o grupo controlo, com significância estatística nos dias 7, 9 e 31 após a eclosão (DAH). A administração deste probiótico diminuiu a heterogeneidade do grupo, com diferenças estatisticamente significativas nos dias 20 e 45 DAH. Estes resultados demonstraram que, com a administração deste probiótico, é possivel diminuir o maneio durante a calibração e o comportamento competitivo dos linguados, diminuindo o número de situações de stress e, consequentemente, aumentando a performance de crescimento. Em relação à microbiota intestinal demonstrou-se que os dois regimes de alimentação promovem a dominância de diferentes populações bacterianas nos dois grupos de tratamentos, sugerindo que o Bactocell tem a capacidade de modular a microbiota intestinal das larvas de linguado.

Palavras-chave: *Solea senegalensis*, linguado, probiótico, Bactocell

I. Introduction

1. Aquaculture overview

Aquaculture is defined as the production of aquatic organisms including fish, molluscs, crustaceans and aquatic plants. The farming of the aquatic species implies the human intervention in the rearing process, in order to increase its production. Unlike fishing, this activity is used to selectively increase the production of species used for human consumption, in industry or in sport fishing (DGRM, 2013; FAO, 2015). The over-exploitation of wild stocks and the increasing world population, allowed the aquaculture to be an important economic activity (Martínez Cruz *et al.*, 2012). Sustainable aquaculture reduces pressure on wild resources and offers a response to increasing demand for marine products (FAO, 2015).

In recent times, the production of aquatic animals has contributed to global food production, materials for both industrial and pharmaceutical areas, as well as on the ornamental market (Martínez Cruz *et al.*, 2012).

The *World Aquaculture 2014 report* found that the world production of farmed fish was around 66.6 million tons in 2012 (as represented in Figure 1), with an annual average growth rate of 6.2% between 2000 and 2012. Additionally, the report also estimated that in 2030, more than 65% of the supply of fish for human consumption will come from aquaculture.

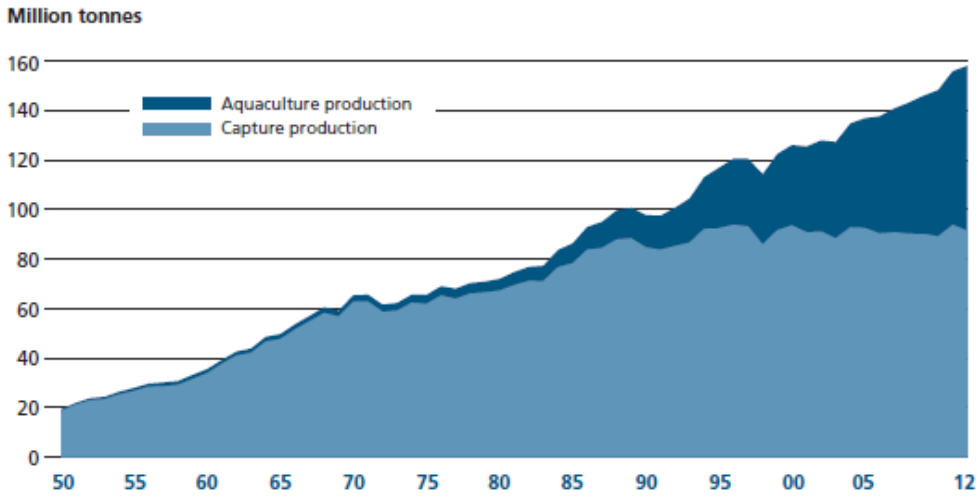

Figure 1 - World capture fisheries and aquaculture production, adapted from FAO (2015).

FAO also reported that 63% of world production in aquaculture are fish, followed by the production of crustaceans (22.4%). Most producers of fish industries are in Asia, responsible for 88% of world production, followed by America and Europe (FAO, 2015).

The European Union (EU) is the 8th largest aquaculture producer in the world, with a share of 1.53%, while China is the main producer with 60.75% in terms of volume. It is

estimated that aquaculture volume production will exceed fishing as the main source of aquatic organisms in a few years (see Figure 2) (Fisheries - European Commission, 2014). The EU annually produces about 1,25 million tons of aquatic species (1.53% of the total global volume production), being 50% of this production, molluscs and crustaceans, 27% marine fish and 23% freshwater fish (Figure 2). European countries such as Spain, France and the United Kingdom stand out on aquaculture production, and the main species produced in the EU are mussel (*Mytilus edulis*), rainbow trout (*Oncorhynchus mykiss*) and salmon (*Salmo salar*) (Fisheries - European Commission, 2014).

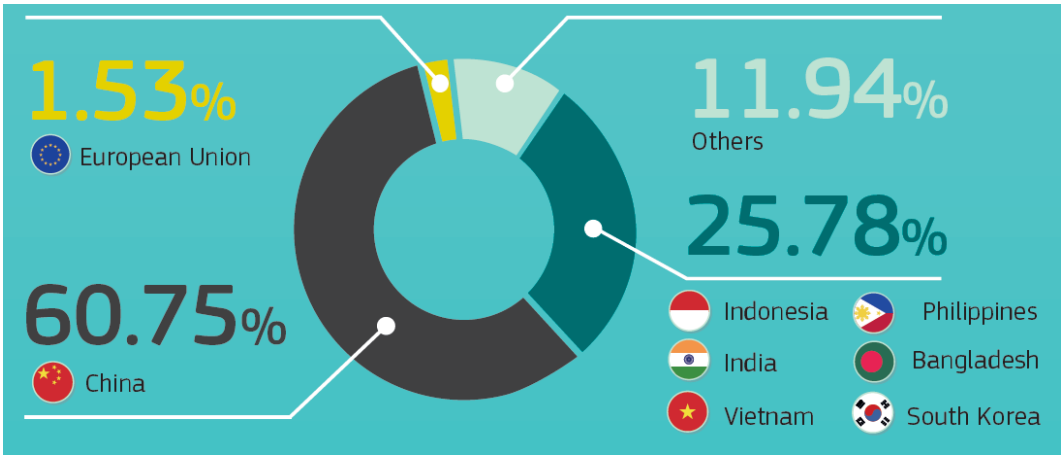

Figure 2 - Distribution of aquaculture volume production in different countries, adapted from Fisheries - European Commission (2014).

Actually, in Europe, the average annual consumption of marine products is about 23.1 kg per person, taking into account that about 24% (5.54 kg) of the consumption comes from aquaculture. The most consumed aquaculture species in the EU are salmon and mussel and 9/10 mussels eaten in the EU are farmed (Fisheries - European Commission, 2014).

In Portugal, the contribution of aquaculture to supply fish is low, 9,160 tons in 2011. Of this total, about 87.9% are organisms produced in salt and brackish waters and 12.1% in freshwater. Aquaculture in Portugal focuses mainly on clam (*Ruditapes decussatus*), mussel (*Mytilus edulis*), seabream (*Sparus aurata*), seabass (*Dicentrarchus labrax*), oyster (*Crassostrea sp.*) and rainbow trout (*Oncorhynchus mykiss*) (DGRM, 2013; INE, 2014). However, in this country only 3% of fish consumed comes from aquaculture, whereas in Europe this value increases to 50% (Fisheries - European Commission, 2014).

The aquaculture activity can be performed in three different regimes according to its productive capacity and type of fish produced: extensive, semi-intensive and intensive. In the extensive system of aquaculture, feeding is exclusively natural and the productive capacity is low; the semi-intensive system joins the natural and artificial food supplements

increasing the production capacity and in the intensive regime the feeding is artificial and the production capacity high (DGRM, 2013; INE, 2014). Regarding the type of exploitation in Portugal, production in freshwater is entirely carried out intensively, while in brackish and marine waters 43.5% are produced in extensive (mainly for the cultivation of bivalves), 45.3% intensively and the remaining 11.2% come from the semi-intensive system (INE, 2014).

It is important to point out other important benefits of this business branch such as the generating of about 85,000 jobs in 14,000 places (90% are micro-enterprises) in EU (Fisheries - European Commission, 2014).

The growth rate displayed by the aquaculture industry is due to the need to find an alternative to the exploitation of wild stocks, since many fisheries have reached their maximum sustainable exploitation. The demand for high quality, healthy, safety and low-calorie food led consumers to consume food with high content of proteins, for instance aquatic products. Fish are a very important factor in human nutrition since they are rich in oils (omega-3), proteins and minerals (Timmons, 2002; Martínez Cruz *et al.*, 2012). In Europe, aquaculture complies with health and hygiene standards, taking into account the animals, consumers and the environment. It also tracks each stage from egg to plate, transmitting the security searched for the consumer (Fisheries - European Commission, 2014).

Today, aquaculture is a profitable industry (Timmons, 2002; Cressey, 2009), being one of the most important food production industries of high nutritional content. Aquaculture has contributed to the welfare of the population, representing a major source of wealth and a livelihood, being one of the possible solutions to the increase of human population density (FAO, 2015).

Aquaculture is now the fastest growing sector of food production in the world (Ige, 2013; Pandiyan *et al.*, 2013). Due to increased human population and stabilization of catches in fisheries, this activity has been moving in different directions, intensifying and diversifying the production (Bondad-Reantaso *et al.*, 2005; Tuan *et al.*, 2013). However, the increase in production and marketing of aquatic products leads to the emergence of many obstacles, such as diseases and epizootics, the need to improve the technique of reproduction, hatching and growth, development of appropriate feed and feeding mechanisms, and management of the water quality (Subasinghe *et al.*, 2003). The feed is responsible for about 70% of the operating costs in most fish farms (Muzinic *et al.*, 2004), so there is a need to develop appropriate quality feeds and feeding methods, in order to improve growth and feed efficiency (Tuan *et al.*, 2013).

In addition, the high density cultivation used in some facilities of intensive aquaculture has caused adverse effects on the environment (organic waste dump),

originating toxic compounds, such as ammonia and nitrate (Kesarcodi-Watson *et al.*, 2008; Martínez Cruz *et al.*, 2012).

Nowadays, animal diseases are the main problems affecting the production of aquatic organisms, having a significant impact on the economy of a country (Qi *et al.*, 2009). These epidemics arise mainly due to the presence of stress conditions, poor water quality and incorrect management, leading to a decrease in productivity and heavy economic losses (Bandyopadhyay and Mohapatra, 2009; Martínez Cruz *et al.*, 2012). The *World Bank report* in 2006 estimated an annual economic loss of 3 billion dollars, due to illness. There are different approaches to be considered in order to mitigate the incidence of diseases in aquatic animal's cultivation (Newaj-Fyzul *et al.*, 2007).

To work with this problem, disease prevention and control has mainly been done with the use of chemicals, for instance antibiotics (FAO/OIE/WHO, 2006; WHO, 2012).

2. Use of antibiotics in aquaculture

As aforementioned, intensive production of aquatic animals exposes the animals to stress conditions, which promotes the emergence of diseases and environmental degradation, leading to economic losses (Pandiyan *et al.*, 2013).

The urgency to prevent and control outbreaks of diseases promoted the use of greater amounts of drug substances. Antibiotics have emerged as an ideal substance, because in addition to controlling bacterial proliferation and decreasing mortality rates, they can be also used as growth promoters. Thereby, antibiotics represented an element of economic interest (Romero *et al.*, 2012).

Growth promoters, as the name implies, are used to improve the growth performance of animals. The use of antibiotics as supplements in animal feed showed benefits for livestock, focusing on the improvement of weight gain and feed conversion (Aly *et al.*, 2008a; Ignatova *et al.*, 2009; Soleimani *et al.*, 2010; Veizaj-Delia *et al.*, 2010).

Antibiotics have been used for decades (Rahiman *et al.*, 2010). However, in recent years there have been some concerns relating to public health and the environment and their use decreased (Balcázar *et al.*, 2006). Until today, tons of antibiotics have been produced and distributed. For example, in the United States were produced about 18,000 tons annually of antibiotics for medical and agricultural purposes, with about 13,000 used for non-therapeutic treatments of cattle (growth promoters) (Balcázar *et al.*, 2006).

As a result of the expansion of these quantities in the world, it is possible to foresee the selective pressure of resistant bacteria which can adapt to different environments, by a horizontal flow of resistance genes (Mohapatra *et al.*, 2013). Resistant bacteria arise mainly through chromosomal mutations or plasmid transfer between organisms (Sharifuzzaman

and Austin, 2009; Pérez-Sánchez *et al.*, 2014). The use of these substances in the prevention and control of animal diseases can cause risks to public health, by promoting the selection, spread and persistence of resistant bacteria (Mohapatra *et al.*, 2013; Pérez-Sánchez *et al.*, 2014). Some cases of resistant bacteria difficult to control and eliminate, mainly due to the indiscriminate use of antibiotics, have been reported previously (Aoki, 1975; Aoki *et al.*, 1984; Miranda and Zemelman, 2002; Vine *et al.*, 2006).

The emerging concern for human health and the environment led to the restriction of the use of antibiotics in aquaculture in EU countries in 2006 (De Angelis *et al.*, 2006; Ige, 2013). This induced the search for alternative products, allowing in the same way to prevent and control the emergence of diseases and also promote higher growth and weight gain (Panigrahi *et al.*, 2010; Pérez-Sánchez *et al.*, 2014).

The efforts to find alternatives to traditional growth promoters led to a greater interest in probiotics, vaccines and immunostimulants. Thus, the use of probiotics has been seen as a promising alternative to the use of antibiotics in animal production (Pandiyan *et al.*, 2013).

Different studies have shown that the endogenous intestinal microbiota acts as a first line of defense against pathogens in terrestrial and aquatic animals (Gómez and Balcázar, 2008; Balcázar *et al.*, 2010). Actually, there are different studies showing the beneficial effects of the use of this compound as a food supplement in feed for poultry, pigs, cattle, fish, crustaceans, molluscs and amphibians (Gatesoupe, 1999; Aly *et al.*, 2008a; Ignatova *et al.*, 2009; Soleimani *et al.*, 2010; Veizaj-Delia *et al.*, 2010). According to Balcázar *et al.* (2006), probiotics were added in the diet in order to accomplish the balance of the intestinal flora of the animals, preventing diseases and disorders, improving digestibility and, consequently, promoting an increase in the availability and uptake of nutrients, which stimulates the growth performance of animals.

The use of probiotics may favor the idea of friendly aquaculture environment (Abdelhamid *et al.*, 2009), being considered as an alternative to antimicrobial agents (Merrifield *et al.*, 2010b).

3. General perspectives in probiotics

The term probiotic has origin from the Greek words pro and bios meaning "pro-life" (de Vrese and Schrezenmeir, 2008), whose concept has undergone changes over the years. Metchnikoff (1907) was the first to suggest the beneficial role played by some bacteria that were consumed through milk. The term probiotic was added by Lilly and Stillwell (1965) and described a substance produced by protozoa which stimulated the exponential phase of other microorganisms. Later this term was used to describe animal

food supplements that benefit the host by improving the balance of intestinal flora (Fuller, 1989).

Guarner and Schaafsma (1998) suggested that probiotics are live microorganisms that confer health benefits to the host when ingested in sufficient quantities. In 1999, this concept is defined as "microbial cells administered in a way which affects the gastrointestinal tract and remain alive with the aim of improving health" (Gatesoupe, 1999). However, other definitions classified these organisms as food supplements that gives health benefits and higher disease resistance (Lara-Flores, 2011).

The Food and Agriculture Organization (FAO) and the World Health Organization (WHO) defines probiotic as live organisms that when consumed in adequate concentrations benefit the health of the host (WHO/FAO, 2001).

Recently, several studies have focused on developing different strategies in order to manipulate the composition of the intestinal bacterial flora of the host. It is known that this modulation may lead to increased growth rate, more efficient digestion and better disease resistance (Burr *et al.*, 2005; Denev *et al.*, 2009).

This modulation, which uses beneficial bacteria and is performed through supplementary feeding, is seen as a viable therapeutic alternative to antibiotics and other drugs (Tuan *et al.*, 2013). These bacteria, commonly referred as probiotics, are able to colonize and multiply on the intestinal flora with several advantageous effects (Nayak, 2010).

Having this in mind, as mentioned above, it is necessary to pay special attention to quality and feeding methods in order to improve the technical aspects. There are different studies showing that supplementation with probiotics reduce the incidence of diseases and improve the general condition and welfare of animals (Kim and Austin, 2006; Mohideen *et al.*, 2010; Wang and Gu, 2010), providing nutritional and enzymatic digestion advantages, as well as increase the immune response and improving water quality (Qi *et al.*, 2009).

The high costs of some of these substances may be countered by decreasing the general production cost due to the growth improvement and feed efficiency (Peterson *et al.*, 2012).

The improvement in the overall condition of the animal and feed efficiency leads to a decreasing in waste production, which consequently diminishes the degradation of water chemical parameters (Velmurugan and Rajagopal, 2009; Nimrat *et al.*, 2012).

Probiotics generate new opportunities in the management of human health. These emergence compounds are receiving an increasing in both scientific and commercial concern, being used for therapeutic and prophylactic functions and as growth promoters (Ige, 2013; Tuan *et al.*, 2013).

Recently, with the increased study of probiotics, a new concept arises; prebiotics are defined as non-digestible food ingredients that selectively stimulate the growth of certain bacteria or symbiotic (Ringø *et al.*, 2014), allowing colonization and stabilization in the gut of the host (Nayak and Mukherjee, 2011).

The use of probiotics, given the overall beneficial effects, is considered as a potential alternative to the use of some chemical drugs. Their use in animal feed is documented (Chaucheyras-Durand and Durand, 2010; Rigobelo, 2012; Newaj-Fyzul *et al.*, 2014) and, lately several articles have been written about the use of these supplements in aquaculture (see Table 1) (Yanbo and Zirong, 2006; Wang and Gu, 2010; Pérez-Sánchez *et al.*, 2014).

There are differences between the intestinal microbiota of terrestrial animals and aquatic animals, as a result of the environment diversity around them. The gut flora of aquatic animals, unlike the terrestrial ones, reflect the environmental microbiota in which they live and where most bacteria are transient. This is due to the continuous intake of food and water and the microorganisms that they contain (Martínez Cruz *et al.*, 2012; Tuan *et al.*, 2013). Therefore, the stability of the intestinal bacterial colonies on aquatic organisms is linked to external factors (Lara-Flores, 2011).

In aquatic animals, probiotics have been isolated from both exogenous and endogenous origin (Balcázar *et al.*, 2006; Balcázar *et al.*, 2010). In marine species, studies have found that gram negative facultative anaerobic as *Vibrio* and *Pseudomonas* are predominant (Askarian *et al.*, 2012). Furthermore, in freshwater species predominate *Aeromonas, Flavobacterium Pseudomonas* and bacteria of the genus *Bacteroides*, *Clostridium*, *Fusobacterium* (Li *et al.*, 2012; Wu *et al.*, 2012) and *Lactic acid* bacteria are subdominant in fish (Tuan *et al.*, 2013).

The dynamics of the intestinal bacterial populations are very complex and there are many interactions between bacteria and between bacteria and the host (Newaj-Fyzul and Austin, 2014). Nonetheless, after selection of a particular strain, it must have different requirements in order to be designated as a probiotic organism (de Azevedo and Braga, 2012). A probiotic must have a good resistance to stomach acid and bile and pancreatic enzymes, access and ease intestinal colonization, ability to stay alive for a long period of time (transportation, storage), produce antimicrobial substances against pathogens, inability to translocation and not toxic to the host and the environment (Balcázar *et al.*, 2006).

Generally, the species used as probiotics are not pathogenic microorganisms, being lactic acid bacteria the most commonly used, such as *Bifidobacterium, Lactobacillus, Lactococcus, Streptococcus and Enterococcu*s (de Azevedo and Braga, 2012).

Table 1 – Probiotic application in aquaculture, adapted from Martínez Cruz *et al.* (2012).

Benefits	Probiotic	Used in aquatic species	Reference
Growth promoter	*Bacillus sp.* *C. divergens* *Lactobacillus helveticus* *Streptomyces* *L. casei* *Bacillus coagulans*	*Catfish* *Gadus morhua* *Scophthalmus maximus* *Xiphophorus helleri* *Poeciliopsis gracilis* *Cyprinus carpio koi*	Queiroz and Boyd (1998) Gildberg *et al.* (1997) Gatesoupe (1999) Dharmaraj *et al.* (2010) Hernandez *et al.* (2010) Lin *et al.* (2012)
Pathogen inhibition	*Pseudomonas sp.* *S. cerevisiae* *Vibrio alginolyticus* *Bacillus spp.* *Lactococcus lactis*	*Oncorhynchus mykiss* *Litopenaeus vannamei* *Salmonids* *Penaeids* *Epinephelus coiodes*	Spanggaard *et al.* (2001) Scholz *et al.* (1999) Austin *et al.* (1995) de Souza *et al.* (2012) Zhang *et al.* (2011)
Digestibility	*L. helveticus* *L. acidophilus* *S. putrefaciens Pdp11*	*Scophthalmus maximus* *Clarias gariepinus* *Solea senegalensis*	Gatesoupe (1999) Al-Dohail *et al.* (2009) Tapia-Paniagua *et al.* (2012)
Water quality	*Bacillus sp.* *L. acidophilus*	*Penaeus monodon* *Clarias gariepinus*	Wang *et al.* (2008) Al-Dohail *et al.* (2009)
Stress Tolerance	*L. delbrueckii* *Alteromonas sp.* *Pediococcus acidilactici*	*Dicentrarchus labrax* *Sparus auratus* *Litopenaeus stylirostris*	Carnevali *et al.* (2006) Varela *et al.* (2010) Castex *et al.* (2009)
Reproduction improvement	*Bacillus subtilis* *L. rhamnosus* *L. acidophilus, L. casei*	*Poecilia reticulata* *Danio rerio* *Xiphophorus helleri*	Ghosh *et al.* (2007) Gioacchini *et al.* (2010) Abasali and Mohamad (2010)

3.1. Probiotics selection

In aquaculture, the selection of a probiotic must be based on different parameters such as the source, safety of the strain, ability to resist to the harsh environment of the digestive system, capacity to produce antimicrobial components, ability to modulate the immune system and capacity to adhere to the target site. Probiotics should also resist to the industrial processes necessary for the production of food and remain viable in the food and during storage (Pérez-Sánchez *et al.*, 2014).

As described in figure 3, probiotics pass through different stages before they reach the market: first they are isolated from the host animal, secondly its strain is identified and characterized and, finally, they are tested in different parameters such as efficiency and certification (Newaj-Fyzul *et al.*, 2014). For this purpose, one of the most important criteria is to select the source of these microorganisms. Probiotics can be obtained from different sources: aquatic environment (for example water and substrate) (Newaj-Fyzul *et al.*, 2014), skin mucus from animals (Tapia-Paniagua *et al.*, 2012) and also from the digestive tract of animals (Cao *et al.*, 2012).

The microorganisms are selected and identified (*i.e.* selective culture) *in vitro* (Geraylou *et al.,* 2014*)*, and then the pure culture with the colony of interest is exposed to different *in vitro* studies. The *in vitro* studies intend to analyze different parameters, including the pathogen inhibition capacity of the probiotics (antagonism tests), competition for adhesion sites (Chabrillón *et al.*, 2006) and/or for nutrients (Kesarcodi-Watson *et al.*, 2008), resistance to digestive tract conditions (Chabrillón *et al.*, 2006), attachment capacity (Hjelm *et al.*, 2004) and production of other beneficial substances such as vitamins and enzymes (Vine *et al.*, 2006). In *in vitro* studies, pathogenic bacteria can be exposed to isolated probiotics with the objective to observe their behavior, by using antagonism *in vitro* tests (Lamari *et al.*, 2014). However, the results of certain probiotic cannot be extrapolated to other species (Balcázar *et al.*, 2006) and, therefore, it must be taken into account the origin, safety (*i.e.* pathogenicity) and the bacteria's ability to survive the harsh environment of the digestive tract. The selection of probiotics is a critical point, since the use of improper organisms can trigger unwanted effects (Pérez-Sánchez *et al.*, 2014). Autochthonous probiotic has a higher probability of success (Sun *et al.*, 2013), and thereby the selection of the host bacteria may be a method of isolating an effective probiotic (Burbank *et al.*, 2012).

Before starting the *in vivo* studies, *in vitro* studies are also used to optimize supplementation doses and the viability of the strain (Román *et al.*, 2012).

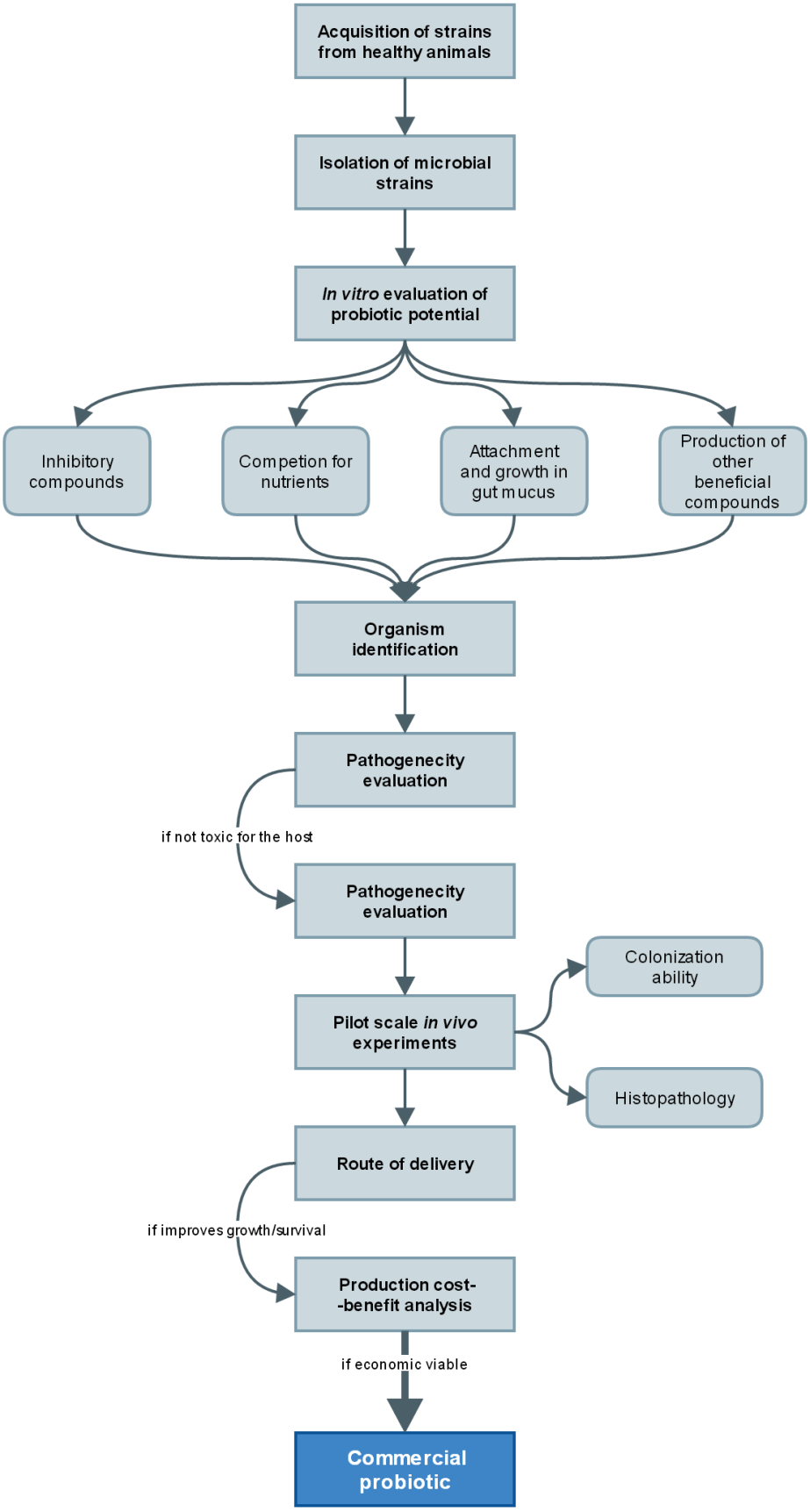

Figure 3 - Diagram of the selection of probiotics, adapted from Balcázar *et al.* (2006).

The last phase consists in *in vivo* evaluation to study the ability to benefit the host (Burbank *et al.*, 2011). For a probiotic to be accepted for commercial purposes, there must be several evidences of its security, adaptability, viability, physiology, genetics, interactions with host and resident microbiota, among others (Kiron, 2012). It is also necessary to do some tests to choose the best route of administration (*i.e.* addition in the rearing water or attachment in the live feed). Finally, after surpassing all the tests including an economic analysis of cost/benefit, probiotics can be produced and marketed (Pérez-Sánchez *et al.*, 2014). The marketing of supplements like probiotics is regulated by different organizations such as the US Food and Drug Administration (FDA) and the European Food Safety Authority, among others. In the EU, the use of bacterial strains belonging to the group *Bacillus*, *Lactobacillus*, *Pediococcus* and *Streptococcus* as probiotics in animal feed has been authorized. However, in Europe, only the probiotic *Pediococcus acidilactici* (strain CNCM MA 18/5M), known as *Bactocell* have authorization to be used in aquaculture (Fisheries - European Commission, 2014).

3.2. Use of probiotics

The early life stages of larvae interfere with the dynamics of microbial populations. Once after the process of hatching, the larvae are in contact with the rearing water, being susceptible to the colonization of different microorganisms (Ige, 2013). During the distribution of first feeds, it is possible to artificially manipulate the settlement of some groups of desired bacteria, by adding them in feeds (Tuan *et al.*, 2013).

Environmental exposure in early life has a significant impact on the gut microbiota composition during the larvae development. It is known that marine fish larvae have a rudimentary gut and few bacteria present at hatch (Vine *et al.*, 2006). Nevertheless, after hatching, their gut colonization begins and the environment provided to the larvae will dictate its adult life (Tapia-Paniagua *et al.*, 2012).

Colonization of the gastrointestinal tract and the establishment and growth of these bacteria occurs after hatch, being necessary to use high doses of probiotics to replace the intestinal flora by the desired strains, with a temporary domain (Balcázar *et al.*, 2006; Pérez-Sánchez *et al.*, 2014). Fuller (1992) showed that after finishing the intake of probiotics, the colony of these microorganisms in the intestinal tract decreased, requiring a prolonged and persistent administration. Some bacteria administered in the feed are retained as part of the gut flora, while other parts are destroyed in the digestive process and the remaining ones are eliminated in the faeces (Pandiyan *et al.*, 2013).

Different factors may inhibit colonization of probiotics, such as body temperature, stress, production of antimicrobial substances by the host (bacteriocins, hydrogen peroxide,

etc.), inhospitable environment (pH, enzymes, etc.), among others (Nayak and Mukherjee, 2011; Pandiyan *et al.*, 2013).

As has been mentioned, colonization capacity and modulation of the host immune system are critical benefits for the use of probiotics in aquaculture (Nayak, 2010), being considered protective mechanisms against pathogenic microorganisms (Martínez Cruz *et al.*, 2012). The ability to compete for adhesion sites and the stimulation of the immune system depend on factors such as the source, viability, dose, duration and administration (Liu *et al.*, 2012).

Bacteria available as probiotics differ in their action mode and its use has different goals (Nayak, 2010). Studies have demonstrated differences between and within species of probiotics in the ability to stimulate the immune system (Pieters *et al.*, 2008) and disease protection capacity (Díaz-Rosales *et al.*, 2009). It must be considered that certain probiotic action differs in different species (Mohapatra *et al.*, 2013).

Probiotics used in aquaculture are predominantly *Gram positive*, especially the group of *Bacillus* and *Bifidobacteria*. Some *Gram-negative* probiotics are also used, for example strains of *Aeromonas*, *Vibrio*, *Pseudomonas* and *Enterobacteriaceae* (Pérez-Sánchez *et al.*, 2014).

Most probiotics applied in aquaculture have terrestrial origin, which might interfere with the success capacity in water, since it may be inefficient, unable to survive or remain viable in an unknown environment (Wang and Gu, 2010). However, isolated probiotic of the same species or the same environment will be more efficient having a higher probability of success, since it is assumed that the host's immune system does not react to these endogenous bacteria (Nayak, 2010).

Studies have shown that the use of a mixture of probiotics with different species is more efficient than a monospecies probiotic, exhibiting different properties of different bacteria. The supplementation of different strains is based on the idea of complementing each other and colonize different niches of the intestinal area, providing several benefits to the host (Newaj-Fyzul *et al.*, 2007).

Most of the probiotics used do not form spores, but are administered as lyophilized preparations. Although spore-forming probiotics have advantages towards the previous ones, once they are heat-stable and can withstand in hostile environments such as the gastrointestinal barrier (Tuan *et al.*, 2013). Among the existing spore-producing bacteria, the *Bacillus* is the most common, mainly due to its stimulant properties of the immune system and ability to improve water quality (Mohapatra *et al.*, 2013).

The majority of the probiotics used in aquaculture are viable, allowing them to adhere and colonize the intestinal surface area (Tapia-Paniagua *et al.*, 2012). However, inactive probiotics can have the similar benefits as the active ones, such as adherence to the

intestinal tissue, stimulation immune responses and controlling of diseases (Borchers *et al.*, 2009). This fact is due to the presence of subcellular and extracellular substances such as capsular polysaccharides and peptidoglycan (Lara-Flores, 2011). Nevertheless, the use of inactivated probiotics would reduce the safety concerns regarding the virulence gene transfer (Newaj-Fyzul *et al.*, 2007).

The optimal dose supplementation is also crucial to allow colonization, proliferation and stabilization of the intestinal flora colony. Notwithstanding, this can be a limiting factor in the successful use of a probiotic in aquaculture (Minelli and Benini, 2008).

In aquaculture, probiotic dose is, generally, between 10^6 to 10^{10} CFU/g feed, and the dose is selected taking into consideration the ability to colonize, promote growth and modulate the immune system, among others. However, the optimal dose varies depending on the host and the beneficial capabilities of the probiotic (Nayak, 2010).

The duration of probiotic supplementation is another factor which promotes the proliferation and stabilization of bacteria and also promotes the immune response. However, the duration of a probiotic supplementation depends on the strain and desirable benefits (Choi and Yoon, 2008). A long diet is beneficial to the host, since it enables the colonization and multiplication of the bacteria in the flora and appearance of beneficial effects (Ige, 2013).

3.3. Probiotic commercial preparations

In the last years, the use of probiotics as an alternative to antibiotics has been increasing and, at this time, there are different commercial preparations containing one or more microorganisms. In 2013, it was estimated that the market value is about 19,600 million dollars (Martínez Cruz *et al.*, 2012).

There are different preparations available in the market and also several technologies to improve the manufacture and use of probiotics. The latter has focused on the improvement and optimization of conditions in order to increase the performance and viability of probiotic (Ige, 2013).

Some products contain prebiotics (*i.e.* glucans, yucca extract, inulin, sugar beet pulp and wheat starch) that increases the effects of the product. Prebiotics are indigestible ingredients that stimulate the growth of beneficial microorganisms, and their use can stimulate the action of probiotics, stabilizing the host gut bacterial population (Smart Microbials Inc, 2012).

The heat applied in the food pellet processing is one of the major stressors agent affecting probiotics (De Angelis *et al.*, 2006). The process to assemble the pellet involves temperatures of 70-80 ºC and the extrusion process may reach values of 90-130 ºC (Svihus

et al., 2010). The majority of probiotics does not withstand these high temperatures, but *Bacillus* species, especially the spores, remain stable during processing and storage (Simon, 2010). However, new techniques have been developed to solve the problem, such as the microencapsulation of bacteria, that permit accumulate the probiotic bacteria in high densities (Rokka and Rantamäki, 2010). There are different methods of microencapsulation, for instance emulsion, extrusion, spray drying and adhesion to starch. These capsules have different substances such as alginate, chitosan or pectin, in order to protect the microorganisms, both physically and chemically (Dianawati *et al.*, 2015). The alginate capsules protect bacteria from the acid pH and digestive enzymes, which allows the release of bacteria still intact in the intestine (Kailasapathy, 2015). The lyophilized preparations support storage and transport without suffering significant damage, allowing bacteria to remain stable and viable (Ige, 2013).

3.4. Administration mode of the bacteria strain

Nowadays, in aquaculture, the probiotics may be added in different forms, including the inoculation of the rearing water with live bacteria, suspensions and feed (Carnevali *et al.*, 2014). Many probiotics are applied directly into the rearing water and health and environment surrounding benefits has been documented (Zhou *et al.*, 2010). Nevertheless, dietary supplementation with probiotics is the most effective way for these bacteria to colonize the gut (Tapia-Paniagua *et al.*, 2012), since oral administration (with live feed or inert diet) allows a more effective stabilization of colonies in the intestine and, consequently, stimulation of the immune system with increased protection (Zhou *et al.*, 2010).

The choice of strain, supplementation mode and its duration depends on the culture conditions and the desired effects in immunostimulation, growth enhancement, reduction of disease incidence, among others (Merrifield *et al.*, 2010b).

In larvae culture, it is usual to administer probiotics suspended or in bioencapsulated forms (Balcázar *et al.*, 2006). Rotifers, copepods and artemia are essential in the early days of larval life, since their small size makes them easy to take and digest. These live food may function as probiotic carriers (bioencapsulated) that stimulates the local immunity of larvae, increasing nutrient availability and the digestion capacity (Martínez Cruz *et al.*, 2012).

Treatments with probiotics are preferable in larval stages of aquatic animals, because this stage is associated with different disorders in the intestinal microbiota due to the start of exogenous feed (Ige, 2013).

4. Probiotic application in aquaculture

In intensive farming systems, as already mentioned, high densities and the handling of animals are stressors agents that may lead to decreased production, low feed efficiency rates and the failure of the immune system. These weakened animals are more susceptible to opportunistic pathogens (Pérez-Sánchez *et al.*, 2014). Bacterial pathogens can enter the fish by different routes, including the gills, skin, gastrointestinal tract, being the mucosal adhesion one of the main focus of infection by the pathogen (Guardiola *et al.*, 2014). Therefore, adherence to mucosa is one of the most important factors in probiotics selection, in order to compete with the invading bacteria (Vine *et al.*, 2006).

Actually, consumers are looking for safe, natural and traceable fish products, additives free. Thus, due to the preference for prevention rather than treatment of diseases, probiotics have been used in feed industry, including aquaculture, to control pathogens (Ramos *et al.*, 2015).

Supplementation with probiotics provides several benefits, including improvement of nutritional value, contribution to enzymatic digestion, pathogen inhibition, growth factors, stimulate the immune system and improvement of water quality (de Azevedo and Braga, 2012).

The probiotics modulate the gut flora by suppressing the growth of pathogenic bacteria (Giraffa *et al.*, 2010), increasing resistance to diseases (Merrifield *et al.*, 2010a), producing inhibitory substances that helps prevent the growth of pathogens and their ability to adhere to the intestinal wall. All these factors interfere with the normal route of infection of some pathogens (Ringø *et al.*, 2014).

Several reports had summarized the use of different probiotics in aquaculture. For instance, these studies reported the use of a wide variety of gram positive and negative bacteria, bacteriophages, yeast and microalgae as probiotics administered via oral and rearing water (Gatesoupe, 1999; Tinh *et al.*, 2008; Qi *et al.*, 2009; Mohapatra *et al.*, 2013; Pérez-Sánchez *et al.*, 2014; Ramos *et al.*, 2015). This proved that probiotics, due to its bactericidal effects, have reduced the incidence and duration of diseases (Tuan *et al.*, 2013). Once again it was shown that probiotics can inhibit pathogenic bacteria in *in vitro* and *in vivo* using different mechanisms of action (Balcázar *et al.*, 2006).

4.1. Mechanisms of action

Some authors have studied several methods whereby the probiotics improve the health and welfare of the aquatic animals. It is possible due to the different mechanisms that these microorganisms use, such as inhibition of pathogens, promotion of growth and

digestibility and also the improvement of water quality, reproduction and stress tolerance (Tuan *et al.*, 2013).

The next sections of this review will explore the general mechanisms of action of probiotics described earlier.

4.1.1. Inhibition of pathogens

The competitive adhesion to the wall of the digestive tract and colonization are one of the pathways of action against pathogenic bacteria, allowing competitive exclusion to obtain a stable, pleasant and controlled microbiota (Pandiyan *et al.*, 2013).

This mechanism is based on the formation of a physical barrier, through the connection of these beneficial bacteria to the binding sites in the intestine, thereby preventing colonization of pathogenic bacteria (Korkea-aho *et al.*, 2012). Bacteria exhibit different setting strategies to the binding sites such as passive forces, electrostatic interactions, hydrophobicity and specific adhesion structures, among others (Ringø *et al.*, 2010).

The living organisms combine different mechanisms to compete for resources such as nutrients, space and oxygen (Pérez-Sánchez *et al.*, 2014). Probiotics may protect the host from the pathogens, producing metabolites that inhibit their growth or colonization or competing for resources such as nutrients and space (Vine *et al.*, 2006).

As is already common knowledge, the interactions and relationships between organisms (competition between beneficial and pathogenic bacteria) in the intestinal flora plays a key role in the balance of the microbiota (Merrifield *et al.*, 2010a). Nonetheless, these indigenous communities can be changed by the surroundings and the husbandry practices that can facilitate the proliferation of certain bacterial species. The possibility of modulating the gut flora of the aquatic animal is an instrument for controlling the proliferation of pathogenic microorganisms (Balcázar *et al.*, 2006).

Allied to this colonization capacity, the selected probiotics sometimes produce antagonistic compounds, defined as chemical substances with inhibiting effects (generally named bacteriostatic) and/or toxic effects (bactericidal). Substances, such as antibiotics, bacteriocins (small peptides that break the integrity of the bacterial cell membrane) (Pérez-Sánchez *et al.*, 2014), siderophores, enzymes such as proteases and lipases, hydrogen peroxide (inhibits the growth of gram negative bacteria) and organic acids (lower pH environment), prevent the growth of different pathogens (Merrifield *et al.*, 2010b). In addition to these facts, the lack of available nutrients (used by the beneficial bacteria) acts as a limiting factor in the maintenance of pathogenic bacteria (competition for nutrients) (de Azevedo and Braga, 2012).

It should be realized that these modes of action complement each other. For example, if the only mode of action is the production of antibacterial substances, the pathogen can develop resistance to the compound, resulting in an ineffective treatment. Therefore, a risk assessment of the development of resistance to antibacterial compounds is necessary, in order to ensure a treatment with an effective and stable probiotic (Pandiyan *et al.*, 2013).

The fish immune system consists in two components, the innate system (non-specific) constituted by cellular and humoral elements (comprehend nonspecific cytotoxic cells and phagocytes) (Collet, 2014) and the adaptive system (specific) defined by the humoral and cellular immune response (includes components such as antimicrobial peptides, lysozyme, complement system, lectins, natural antibodies), which is less developed in fish (Mutoloki *et al.*, 2014).

Fish have a relatively simple immune system and it is believed that probiotics have an important role in stimulating the immune response, which is feasible for the inhibition of pathogens and disease control in aquatic organisms (Sfacteria *et al.*, 2015).

Probiotics may modulate the non-specific immune system of aquatic animals, increasing its resistance to disease. The liposaccharides, peptidoglycan and B-glucan present in the bacterial walls of probiotics stimulate the innate and adaptive immune system (Pérez-Sánchez *et al.*, 2014). The administration of certain bacterial strains can promote increased phagocytic and lysozyme activity (Mandiki *et al.*, 2011; Ridha and Azad, 2012) production of lymphocytes (Aly *et al.*, 2008b), leukocytes (Sharifuzzaman and Austin, 2010; Korkea-aho *et al.*, 2012), the cellular "respiratory burst" (Sharifuzzaman and Austin, 2009; Zhou *et al.*, 2010), production of acid phosphatase and antimicrobial peptides (Balcázar *et al.*, 2010), increased complement activity (Harikrishnan *et al.*, 2010; Sun *et al.*, 2013) mucosal and systemic antibodies production (Korkea-aho *et al.*, 2012; Ridha and Azad, 2012), among others, in response to invading pathogens.

Besides, probiotics are able to modulate the production of cytokines that are protein mediators produced by the immune cells that promote cell growth, differentiation and activation of host defense mechanisms (Nayak and Mukherjee, 2011).

Studies have shown an increased innate immune response with the use of probiotics in different species, such as *Lactobacillus belbrueckii* in seabream (de Azevedo and Braga, 2012), *B.subtilis* and *Pseudomonas aeruginosa* in Indian carp (Giri *et al.*, 2012), *Lactococcus lactis* in tilapia Nile (Zhou *et al.*, 2010) and *Bacillus* and *Vibrio sp*. in the white shrimp (Balcázar *et al.*, 2010). The administration of *S. putrefaciens* and *Shewanella baltica* in *Solea senegalensis* increased the "respiratory burst activity" of leukocytes. Some authors demonstrated that Pdp11 (*Shewanella sp*.) inhibited the *in vitro* growth of *P. damselae*

subsp. *piscicida* and some virulent strains of *V. harveyi, V. anguillarum* and *V. alginolyticus*.(Tapia-Paniagua *et al.*, 2012).

In summary, probiotics can inhibit the pathogens proliferation by antibiosis, competition for nutrients and space, modulation of microbial metabolism and the stimulation of the immune system of the host (Tuan *et al.*, 2013).

Merrifield *et al.* (2010b) showed that these benefic microorganisms can also inhibit virus gene expression due to the release of different chemical and biological substances. A study showed that strains of *Pseudomonas sp*., *Vibrio sp*., *Aeromonas sp*. isolated from salmon hatcheries have shown antiviral activity against the infectious hematopoietic necrosis virus (IHNV) (Pandiyan *et al.*, 2013).

Certain species of phytoplankton are also capable of producing toxic substances to other bacteria. The *Skeletonema costatum* is a microalgae used in shellfish hatchery that produces substances capable of inhibiting the growth of *Listonella anguillarum* and other vibrio (Kesarcodi-Watson *et al.*, 2008).

Likewise, yeasts can be used as probiotics, promoting the growth and stimulating the immune system of fish. Once yeasts are not affected by antibiotics, they can be used for recover the normal microbiota after an antibiotic treatment (Pérez-Sánchez *et al.*, 2014). The use of *S. cerevisiae* in rainbow trout improved their growth due to amino acids which increase the palatability and digestibility of trout (Sheikhzadeh *et al.*, 2012).

4.1.2. Growth and digestion promoter

Probiotics have been used to improve the appetite and digestibility of farmed animals, in order to increase their growth (Mohapatra *et al.*, 2012).

Studies suggested that these strains synthesize extracellular enzymes such as proteases, amylases and lipases that facilitate and benefit the food digestion (Zokaeifar *et al.*, 2012). Moreover, the bacterial activity is able to produce vitamins, fatty acids, among others (Ringø *et al.*, 2014), acting as growth promoters and supplementing the dietary needs of farmed animals (Abdelhamid *et al.*, 2009). Also, secreted enzymes break the peptide bonds and produce free amino acid, facilitating absorption (Mohapatra *et al.*, 2012). These substances promote an increase in feed efficiency, higher growth, decrease the incidence of eating disorders and increased survival rate. Consequently, the digestive balance, digestibility and nutrient absorption are improved (Tuan *et al.*, 2013). Thus, since probiotics allow an improvement in nutrient digestibility feeding costs in aquaculture may decrease, once the feed rate increases and food waste decreases (Zokaeifar *et al.*, 2012).

Different strains of probiotics have been reported as growth and survival promoters. For example, the use of probiotic *Streptococcus spp.* in Nile tilapia (*Oreochromis niloticus*)

increased the crude protein and lipids in fishes. After 9 weeks of cultivation, the weight increased from 0.154 to 6.164 g (Martínez Cruz *et al.*, 2012).

In ornamental fish (for example *Xiphophorus helleri*, *Poecilia reticulata, P. sphenops* and *X. maculatus*) it was also reported an increase in the growth and survival when supplemented with *Bacillus subtilis* and *Streptomyces* (Dharmaraj *et al.*, 2010).

Diets supplemented with Pdp11 promoted increased growth of larvae and juveniles of Senegalese sole, increasing muscle protein content (Tapia-Paniagua *et al.*, 2012).

4.1.3. Improvement of water quality

Some probiotics have the ability to improve the water quality in the cultivation of aquatic organisms, modifying the microbial composition of water and substrate. The increase in organic load (nitrogen compounds - ammonia, nitrite and nitrate) are a growing environmental concern in aquaculture (de Azevedo and Braga, 2012). The use of probiotics can reduce the accumulation of particulate and dissolved organic matter, decreasing the concentrations of nitrogen compounds and phosphate (Tuan *et al.*, 2013).

It is important to highlight the role of bacteria *Bacillus sp*. associated with the improvement of water quality. It is a gram-positive bacterium that converts organic matter into CO_2, being possible to decrease the concentration of dissolved and particulate organic matter. The increase in CO_2 production, also allows a stable growth of phytoplankton (Martínez Cruz *et al.*, 2012).

Probiotics used to improve water quality must have some requirements such as the ability to decompose organic matter, reduce the concentrations of nitrogen and phosphate compounds, enhance the growth of phytoplankton, increase the availability of oxygen, suppress blooms of cyanobacteria and other malign microalgae, reduce incidences of disease and increase survival and production (Martínez Cruz *et al.*, 2012; Tuan *et al.*, 2013).

4.1.4. Stress tolerance

As aforementioned, aquaculture tends to focus in intensive production, in order to cover the global food needs, taking into account the increase of human population (FAO, 2015). Such practices, however, due to the high density cultivation, may cause stress in the farmed animal decreasing appetite, feed conversion ratio and increasing the incidence of disease and eating disorders. These changes have a direct impact on animal growth and performance (Pérez-Sánchez *et al.*, 2014).

Probiotics can be used to increase stress tolerance, decreasing cortisol levels and improve growth. For instance, the supplementation with *Lactobacillus delbrueckii* in the diet of seabass (*Dicentrarchus labrax*), at intervals of time between 25 and 59 days promoted reduction of cortisol levels in the test group, which are significantly lower compared to the control group. It was also observed an improvement in seabass growth (Carnevali *et al.*, 2014).

Another study with seabream (*Sparus aurata*) used the plasma glucose and lactate as stress markers (such compounds increases as a secondary response to stress to cover energy gaps) and assess the levels of these substances in the control group and in the group supplemented with *Alteromonas sp*. strain Pdp 11. They concluded that the reserves of glycogen and triglycerides in the liver were significantly lower in the control group (Varela *et al.*, 2010).

It was also reported that supplementation with *L. fructivorans* and *L. plantarum* increased stimulation shock protein (HSP) 70 in gilt-head bream, increasing the stress resistance (Rollo *et al.*, 2006).

Other authors confirmed that the use of probiotics decreases the effects of stress in fish. Thereby, prophylactic treatment with probiotics that precede stressful procedures such as the transportation, husbandry, and change of physical-chemical parameters such as water temperature, among others, can decrease the incidence of eating disorders and diseases (Pérez-Sánchez *et al.*, 2014).

4.1.5. Reproduction

The breeding of aquaculture species has challenging nutritional requirements, being fertilization and fertility as well as the quality of eggs and larvae dependent on the concentration of lipids, proteins, fatty acids, vitamins (C and E) and other substances (Izquierdo *et al.*, 2001).

In hatcheries, the broodstock are fed with commercial diets and fresh products such as squid, cuttlefish and small crustaceans. Adding probiotics to the breeders feed can prevent the incidence of diseases and parasites (Martínez Cruz *et al.*, 2012). Furthermore, studies in ornamental fish shown that the use of certain probiotics can increase gonodosomatic index, fecundity and viability of eggs and larvae quality (Ghosh *et al.*, 2007). These authors also suggested that vitamin compounds such as thiamine (B1) and vitamin B12 help to reduce the mortality rate and fry deformations.

II. Introduction to the aquaculture facilities

In Portugal, the growth of marine aquaculture industry is assigned to a small group of species, whose market has become saturated (for example gilt-head bream (*Sparus aurata*) and sea bass (*Dicentrarchus maximus*)). In order to diversify the cultured species, the sole culture (*Solea senegalensis*, Kaup 1858) emerges as a promising species in southern European countries (Morais *et al.*, 2014a), being an autochthonous species of great commercial interest (Imsland *et al.*, 2003).

The Senegalese sole is a flat fish with a high commercial value that drew the attention of investors in southern Europe. The sole high demand in Europe and the fact that this demand is not being fully satisfied by fisheries, led to the increase of its production (Morais *et al.*, 2014a).

Solea senegalensis was chosen for cultivation in southern Europe countries, since this species presents the highest growth rates when compared with the *Solea solea* (Howell et al., 2011).

Due to marine resources over-exploitation, the sole fisheries declined by 43% from 1995 to 2012 and the average size of sole caught also decreased but the average sole prices has remained stable from 2002 to 2013, according to MercaMadrid (fish markets in Spain) (Bjørndal and Guillen, 2014).

The production of flatfish increased from 26,300 tons in 2000 to 148,800 tons in 2008, being China the largest producer in the world and Spain the major producer in Europe (FAO, 2015). According to EUMOFA, from the 7,752 tons of flatfish produced in Europe in 2011, 157 tons were sole (Morais *et al.*, 2014a).

The sole production in Southern Europe has increased significantly from 60 tons in 2005 to 194 tons in 2012 in Spain and 11 tons to 100 tons in Portugal. In 2013, a production of 343 tons of sole and 3,9 million of sole juveniles was registered in Spain (FEAP, 2012; APROMAR, 2014). The farmed sole production in Portugal, Spain and Portugal between 2006 and 2013 can be observed in the Figure 4.

Currently, the sole production costs were estimated in € 9.62/kg. Nevertheless, it is expected a decrease in the costs with the increase of cultivation and improvement of the husbandry techniques. In MercaMadrid, in 2013, the cultivated sole with about 500 grams reached the value of 12.25 €/kg. However, larger fish are preferred by consumers that can afford higher market values (Bjørndal and Guillen, 2014).

The sole production has always been associated with salt marshes (extensive/semi-intensive production) in southern Spain and Portugal in polyculture systems with the seabream and seabass culture (Ferreira *et al.*, 2010). Nowadays, the tendency is the use of intensive farming system (fiberglass or shallow raceways), with controlled environments and commercial foods (Imsland *et al.*, 2003). In order to promote a sustainable aquaculture, improve water quality and control environmental conditions, recirculation aquaculture

systems (RAS) have been implemented in most intensive aquaculture in Spain and Portugal (Morais *et al.*, 2014a).

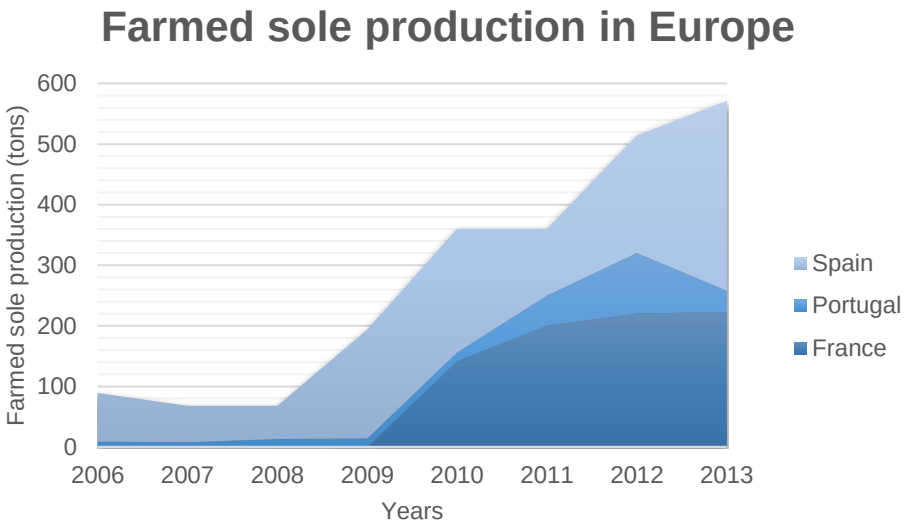

Figure 4 - Farmed sole production in Europe, between 2006 and 2013 adapted from FEAP (2012).

The production of this species has been intensified in recent years in Portugal (Morais *et al.*, 2014a), mainly due to the conversion of an old turbot fish farming to a current and reformulated sole hatchery situated in the north of Portugal.

The company “Safiestela - Sustainable Aqua Farming Investments, SA” is an intensive unit of sole production in Estela – Póvoa do Varzim. The “Safiestela” and “Aquacria Piscicolas, SA”, located in Torreira, belongs to the spanish group called Sea8. The group’s goal is the mass production of juveniles in their unit in Safiestela, followed by the on growing phase in Aquacria. The production starts in Safiestela where the reproduction and the growing of the larvae until the ongrowing phase is performed and then the sole juveniles are transported to Aquacria, where it will grow to market size.

There has been some technical improvements and advances in knowledge, which comprise the breeding, behavior, physiology, nutrition, immune system modulation in response to stress, which has allowed the company to be competitive and sustainable (Morais *et al.*, 2014a). Safiestela is a hatchery comprising different sections: holding of breeders, incubation rooms, larval development room, weaning room, pre-ongrowing room and live food cultivation room.

1. Sole broodstock facilities

Eggs used in the production of sole are obtained from wild broodstock kept in captivity (Figure 5) (Martín *et al.*, 2014). These are preferably obtained from coastal areas in the south of Portugal and Spain (obtained from fishing activities) or from fish farms. Adult sole can be caught using pots, drag or by hand when the earth ponds are emptied. To minimize the stress caused in the fish, it should be taken a special care to the collection and transport of these specimens in order to not damage the animal, since it can compromise their adaptation and breeding in captivity.

Fish are transported to the aquaculture facility and, after a period of quarantine and acclimation in captivity, wild broodstock (more information about wild *Solea senagelensis* can be found in the appendix) can spawn naturally (breeder characteristics can be seen in Table 2) (Imsland *et al.*, 2003). It is described that wild breeders in captive have spawned one year after capture (Dinis *et al.*, 1999).

Figure 5 - Sole broodstock in a circular tank (picture taken in Safiestela).

The aquaculture industry uses wild broodstock (Figure 5) because eggs obtained from captive-bred animals are not fertilized (Carazo, 2013; Morais *et al.*, 2014b). However, there are concerns about the dependence of the capture of wild broodstock for the cultivation of the species, since its stock has been declining due to over-exploitation and is becoming difficult to find suitable adults for reproduction (Morais *et al.*, 2014a).

The captured animals are submitted to a period of quarantine and subsequently analyzed and tagged with microchips, which enables the registration of the individual total length, total weight and sex of soles. Sex is determined by palpation of the gonads or hormonal blood tests (the presence of vitellogenin in 400 grams or larger fishes is an indicative factor of a female). The microchips allow the identification of the individual and

the follow-up of the condition index during the process of maturation. During the maturation process, periodically, a sampling can be carried out, in order to observe the evolution of breeders (weight, length and sexual maturation). The weight and length allow the calculation of the condition index. This parameter allows the characterization of the fish species condition in relation to the weight and length, and it should be superior to one (Dinis *et al.*, 2007).

Table 2 - Sole breeder characteristics, adapted from Howell *et al.* (2011) and Martín *et al.* (2014).

Broodstock	
Female mean weight	1.1-2.2 kg
Male mean weight	0.9-1.5 kg
Density	0.6-4.6 kg/m^2
Male/Female ratio	0.7-2.3
Water Exchange	300%/day
Temperature	11-22 ºC

Breeders are selected by color and body shape, lack of deformations and diseases.

During the quarantine period sole adults are fed with live food (polychaete worms, molluscs) to facilitate their adaptation to the new environment and the inert feed is introduced. The breeders are kept in groups with a male/female ratio of 1:1 or 2:1, to promote fertilization, in square tanks with dimensions higher than 3 m^2 with a water column about 80 cm (holding tanks conditions can be observed in Table 3). However, larger volumes gave higher fecundities (Howell *et al.*, 2011).

Table 3 - Holding conditions of Senegalese sole, adapted from Imsland *et al.* (2003).

Holding tank	
Water depth	0.7-1.4 m
Volume	3-28 m^3
Photoperiod	Natural

In Safiestela, there are 4 different rooms with different groups of breeders (3-4 tanks), each of them independently manipulated for temperature and photoperiod. Each of the rooms has the maturation period at different seasons (autumn, winter, spring and summer) in order to have spawning all year round. The maturation and egg collection depends on an annual temperature cycle, which is artificially manipulated. It is possible to

induce spawning between 16 and 23 °C, ensuring the continuous sole production (egg production characteristics can be seen in Table 4) (Anguis and Canavate, 2005).

The food is based on dry and live food (polychaete worms, mussels, squid), about 2-4% of wet weight of the tank.

The amount and type of food to supply depends essentially on temperature and the number of animals. For animals at lower temperatures, less amount of food is provided, while to active and sexually mature animals a higher amount and variety of food is provided to cover their nutritional and energy needs. Feed with higher amounts of essential fatty acids (EPA and DHA) improve the quality of eggs, sperm and larvae (Duncan *et al.*, 2013; Beirão *et al.*, 2015).

Table 4 - Egg production for natural spawn of captive wild sole, adapted from Martín *et al.* (2014).

Egg production	
First Spawning	April-May
Duration	4-36 weeks
Spawning temperature	16-21 ºC
Mean fertilization	73%
Mean hatching	61%
Daily fecundity (eggs/kg)	734-34,874

In this section, the daily management comprises the analysis of the physical-chemical water parameters (temperature, salinity, dissolved oxygen, ammonia and nitrites), tank cleaning, removal of uneaten food, cleaning and disinfection of the floor.

During spawning season, after collection of eggs, the egg collector should be cleaned and disinfected. Other equipment is also checked, as water pumps, UV filters, heat exchangers and sand filters (backwash cleaning, by reversing the water route in order to unpack the sand and removing organic and inorganic matter accumulated).

2. Egg incubation room

Eggs are obtained by natural fertilization from the wild population held in captivity, since the artificial insemination is complex because it is difficult to manually extract (due to the abdominal pressure) the female oocytes and the male sperm (Cabrita *et al.*, 2006).

Spawning and natural fertilization of the eggs occurs at night, and the fertilized eggs are collected in the morning. At the water exit on the surface of each tank there is an egg collector with a mesh of 400 micrometers (µm). Sole eggs have a diameter of about 800 µm

and has a resistant chorion. A viable egg has to be transparent and have lipid droplets (see Figure 6), while an unviable egg is usually opaque (Imsland *et al.*, 2003).

Figure 6 - Sole viable egg with lipid droplets.

Prior to initiation of the incubation of eggs, unviable eggs should be discarded, in order to avoid putting non-viable eggs in the incubator that may deteriorate water quality. Therefore, it may be used a method which consists in separating eggs in a cylindrical container with salt water (around 32-35 ‰ of salinity), where unviable eggs sink in the water column and viable float. It is also possible, using this method, to calculate volumetrically the amount of collected eggs (where 1 mL corresponds to about 1,500 eggs) (Dinis *et al.*, 2007; Neufeld *et al.*, 2011).

The eggs are incubated in conical tanks with an open water system (Figure 7) with a temperature of about 20°C, with a density of 1,000-10,000 eggs per liter that hatch after 48 hours (± 800 degree-days) (Dinis *et al.*, 2007).

Batches are characterized by the fertilization rate (number of fertilized eggs in relation to the number of eggs obtained) and the hatching rate (number of larvae obtained after hatching in relation to the number of fertilized eggs), allowing to estimate the viability of these spawning.

Figure 7 - Egg incubation room at Safiestela.

3. Larvae culture

In many saltwater species the larval stage is the most critical of the production cycle, due to the high incidence of mortality and deformations. Metamorphosis and the weaning of live food to inert food are the most critical phases of the larval stage of flat fishes (Morais *et al.*, 2014a). Nevertheless, the post-larval and larval rearing of Senegalese sole has less counterproductive problems when compared to other marine species and other flat fishes. For this reason, culture protocols were established and, nowadays, these are standardized and the larvae are produced with good growth and high survival rates (rearing tanks in Figure 8) (Imsland *et al.*, 2003).

Figure 8 - Larvae rearing tanks with 3 m^3 of water.

In the ongrowing of sole, conditions must be adapted to their nutritional needs and their behavior (larvae culture conditions in Table 5), because it is an animal initially pelagic and after metamorphosis becomes benthic and this transformation affects the behavior, feeding and digestive physiology. Therefore, some problems that arise in the hatchery and growth of this species (*i.e.* weaning, deformations, different growth rates, disease), can be improved with the knowledge of physiology and nutritional requirements of the larvae (Morais *et al.*, 2014a).

The marine fish larvae have reduced dimensions and a rudimentary digestive system and photoreceptors (visual sensory organs) compared with freshwater fish (Figure 9). These have specific nutritional needs, being necessary to administer live prey that should be appropriate to the size of the mouth of the larva and its predation mechanisms, in order to facilitate the detection and capture of the live food (Vine *et al.*, 2006).

Table 5 - Characteristics of sole larvae culture tanks, adapted from Imsland *et al.* (2003).

Holding tank	
Water depth	0.7-1.4 m
Volume	3-28 m^3
Temperature	18-20 ºC
Larvae density	± 15/L
Photoperiod	16L:8D
Dissolved oxygen	90-100%
Salinity	35-38 ‰
Ammonia	<0.005
Nitrites	<0.002

Nowadays, it is common to add to the culture water green microalgae (*Tetraselmis suecica* and *Nannochloropsis sp*.), a technique called green water. Microalgae increase the contrast of live prey facilitating their predation; they are a component in the larval diet and also contribute to the nutritional enrichment of live prey (Imsland *et al.*, 2003)

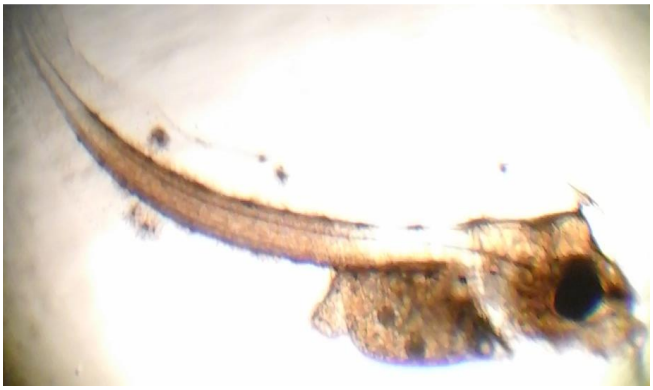

Figure 9 - Sole larvae with 3 mm.

This is a critical phase in larval development, since it requires the acceptance, adaptation and learning of the capture of prey. Mortalities at this stage are due to the adaptation process to exogenous food, but may also be due to poor diet, taking into account the size, nutrient quality and quantity of food (Vine *et al.*, 2006). During the pelagic stage, the larvae exhibit a diurnal behavior, while in the benthic phase they became nocturnal despite they can be fed throughout the day (Navarro-Guillén *et al.*, 2015).

The quality of food in larval stage define the incidence of skeletal deformations, skin discolorations and disease (Vine *et al.*, 2006).

The sole larvae produced in aquaculture have a standardized diet (Imsland *et al.*, 2003). The feeding starts with *Brachionus plicatilis* on 3rd DAH (days after hatching) and

remains approximately 5 rotifers per milliliter throughout the day, to facilitate the predation by larvae. Rotifers are an important element in the diet of sole, due to its small size and its easy digestion. They are enriched (by bioencapsulation) with commercial products suitable for the needs of the larvae, in order to increase the concentration of highly unsaturated fatty acids (HUFA) in the rotifers, allowing increased lipid reserves in the larvae. For example, docosahexaenoic acid (DHA) is very important for the brain, vision, pigmentation and metamorphosis of the larvae in development (Lobo *et al.*, 2014b). In the 5-6th DAH, larvae are usually fed with newly hatched artemia (*Artemia salina*). These artemia nauplii do not feed and, therefore, their nutritional quality cannot be changed. On 7-8th DAH, most larvae consume predominantly artemia, so the feeding of rotifers is interrupted. Between 8-10th DHA feeding starts with artemia metanauplius that are enriched for 24 hours to increase its nutritional quality. By the 9-12th DAH, when the larvae feed preferably with artemia metanauplius, the feeding with small artemia nauplii ceased.

The metamorphosis begins eleven days after hatching and ends on the nineteenth day (19th DAH) (Imsland *et al.*, 2003). Until the metamorphosis is completed, larvae are kept in cylinder conical tanks with 2,800 liters that can support up to 100,000 larvae and, after the settlement, they are moved to circular tanks with about 10 cm of water column (called the weaning area).

Feeding during the production cycle starts in the morning and, during the day, some samplings of the live food concentrations on the tank are made, in order to adjust to the desired prey density.

The physicochemical and environmental parameters should be kept constant and the management should be avoided because the larvae are sensitive to sudden changes of parameters. The metabolites resulting from excretions and dead animals are the main factor of change in water quality, so it uses an open water system to avoid higher concentration of these metabolites in the production tanks. The water in the culture system is treated with mechanical filtration (*i.e.* sand filters, cartridge) and ultraviolet radiation.

The parameters, daily monitored, are based on temperature, oxygen, salinity, pH, ammonia and nitrite. Ammonia and nitrite are toxic nitrogen compounds that, if present, can increase mortality in intensive production systems.

The daily activities in the larval rearing room comprise the control of water quality parameters including pH, oxygen, temperature, chlorine, ammonia and nitrites. At this stage, larvae are kept in open flow system and the water from the sea is firstly filtered (using sand filter, cartridge) and disinfected using UV lights.

4. Live food

Rotifers are produced in continuous batch culture, using cylindrical tanks (see Table 6).

Table 6 - Conditions of rotifers culture, adapted from Imsland *et al.* (2003).

Holding tank	
Volume	2-10 m^3
Photoperiod	24 hours of light (hL)
Dissolved oxygen	80-90 %
Salinity	24 ‰
Temperature	28 ºC

They are fed with a mixture of algae and yeast and enriched the day prior to administration to the larvae with a supplement rich in fatty acids, in order to increase its nutritional quality.

On 3rd day of growth, the rotifers are filtered and their concentration is checked. Part of the rotifers are enriched for later administration to the larvae and another part will be used to start a new cycle (Ferreira *et al.*, 2010).

The artemia is an organism that lives in the water column and is rich in proteins, vitamins and minerals, being a suitable food for the sole larvae (Gisbert *et al.*, 2014).

According to the amount needed to feed the larvae, artemia eggs are placed in water 48 hours prior to the feeding of larvae (conditions of the culture tank can be seen in Table 7). After 24 hours, artemia hatches and is filtered to remove the non-hatched cysts and empty capsules. After filtration, the newly hatched artemia is enriched for 24 hours for subsequent administration to the larvae. The enrichment of artemia consists of adding a supplement rich in fatty acids (bioencapsulation) (Ferreira *et al.*, 2010).

Table 7 - Conditions of artemia culture, adapted from Imsland *et al.* (2003).

Holding tank	
Volume	2-10 m^3
Photoperiod	24 hours of light (hL)
Dissolved oxygen	80-90 %
Salinity	20-35 ‰
Temperature	28 ºC

The pH is kept at 8 using a strong base such as sodium hydroxide (NaOH), called caustic soda.

In the live prey production room, daily routines are based on the oxygen control every 4 hours, tank cleaning, floor and footbaths washing and daily counts for the control of population density.

5. Weaning room

The sole larvae after metamorphosis are transferred to square tanks with a low water column (Figure 10). The low water column facilitates the tank cleaning and the fish observation. Additionally, the residence time of food decreases, reducing nutrient losses and depletion of water quality. It also enhances the availability of live prey to bottom dwelling post-larvae.

Figure 10 - Sole weaning room.

At this stage, some tanks having post-larvae feeding with larvae prey are in open flow system and the water is heated, filtered and sterilized at the entrance. The other tanks having weaned juveniles are in a closed system where sand filters and cartridge are used, as well as a protein skimmer with ozone addition and biological filter, in order to maintain good and stable water quality.

The temperature for sole culture can follow a natural thermoperiod or can be kept at constant temperature between 18 and 20 °C. Although temperatures between 22 and 25 degrees promote higher growth, temperatures above 22 °C increase the risk of incidence of pathologies (Howell *et al.*, 2011).

Sole juveniles are euryhaline and tolerate salinities between 5-55 ‰. However, salinity below 15 ‰ decrease growth compared with a salinity of 25 ‰ (Arjona *et al.*, 2009).

They have a nocturnal pattern of activity after metamorphosis, with a greater activity in the first part of the dark period (Bayarri *et al.*, 2004), and a higher metabolic rate during

the dark phase (Castanheira *et al.*, 2011). The fish farms typically use a 12hL:12hD photoperiod regime and some shading in tanks to maintain light at the surface between 80 and 400 lux (Navarro *et al.*, 2009). In this phase, the larvae are maintained at a density of 3,000 larvae/m^2 and are fed with artemia metanauplius 4 times per day (enriched for 24 hours) until the start of weaning (Imsland *et al.*, 2003).

Weaning of larvae can be done abruptly (fasting day followed by inert food administration) or by co-feeding (larvae fed with artemia and inert food over a period of 7 days (Engrola *et al.*, 2007).

Parameters such as density and average weight of each tank allow calculating the weekly feed conversion ratio, which allows a continuous adjustment of the quantity of food to be distributed in the tank. In order to acquire a homogeneous population (to induce less competition for food and consequently increase fish growth), size grading in the tanks of the same age (same batch) is made regularly. During the size grading process, the fish condition can be analyzed in detail and fish with deformities and diseases can be removed. In this stage, workers should use baskets, whose mesh size corresponds to the size of the fish to select. As mentioned by Morais *et al.* (2014b) the *Solea senagalensis* specie presents a growth rate disparity in individuals with the same age. So, typically selection of smaller sizes is made and slow growers are discarded.

The weaning phase is critical, due to the acceptance problems of inert food by the larvae, which leads to increased mortality and incidence of disease. The incidence of epizootics and higher mortalities, led to the necessity of a regular and rigid control. The temperature is measured and recorded every 4 hours (must be between 18.5 and 20 °C) and the salinity is measured daily with a refractometer. The redox value (oxidation-reduction), oxygen (100% saturation rate) and pH are monitored using sensors. Daily, during the cleaning of the tanks, the general condition of the fish is observed and any illness or damage fish are discharged. The tank is treated according the Sea8 protocol.

Ammonia and nitrite are also measured and recorded daily, as these may vary according to the animal faeces, uneaten feed and biofilter efficiency. It is important to measure these parameters in RAS, being necessary to renew part of the water when these compounds reach toxic concentrations. These measurements are performed by colorimetric methods by using spectrophotometry.

In this room, daily routines are based on the cleaning of tanks (inlet and outlet water pipes), floor and footbaths washing and environmental parameters analysis.

In relation to the filtration system, a "backwash" of the sand filters is made periodically and several parameters are verified such as the silo feeder, the water level in the skimmer, the level of the oxygen tank, pre-filters of the pumps, the biofilters and heat exchangers.

6. Pre-ongrowing sole room

When the fish is about 90 DAH it is graded and transferred to the pre-ongrowing room until it reaches about 40 grams when it is transported to Aquacria, located in Murtosa, for the growing phase until achieves market size. In the on growing, the sole are kept in raceways with dimensions of 12x2x0.20 meters, provided with a low blue light intensity similar to the light that prevails in benthic areas.

At this stage, the larvae already passed the weaning phase and they feed on inert feed efficiently. During ongrowing, commercial diets are the basis of feeding and the daily dose of feed is 3-5% of the total biomass of the tank for this age. To administer the food throughout the day, automatic feeding systems are commonly used.

At the beginning, the pellet has a diameter of 0.75-1 cm and should be increased depending on the size of the fish mouth, ending with a diameter of about 2.5 cm. As in the weaning stage, the amount and the diameter of the pellet calculated in function of the tank density, weight gain and the mean weights.

The cultivation temperature should be around 18-20 °C, salinity between 30-35 ‰ and the oxygen concentration should be greater than 5 mg/L. In on growing stage there are also regular size grading procedures that are done in order to standardize the batch and verify the incidence of disease and deformities in fish (Morais *et al.*, 2014a). This process is done with a size grading machine, which automatically split the fish into the desired size.

As in the larval stage and weaning stage, the water quality is a crucial factor for the development of the fish. In Safiestela, water quality is maintained using a RAS. This system minimizes water exchanges and its purification and sterilization systems ensure good water quality.

The physical and chemical parameters, such as the stages described above, are measured and recorded daily. Daily routines in this room are based on the cleaning of tanks, the water inlets and outlets, washing and disinfection of floor and water foot bath.

At every stage the working equipment and materials must be disinfected with sodium hypochlorite or other disinfectant and physicochemical parameters must be monitored daily and rigorously. Several protocols are followed for this purpose.

7. Recirculation aquaculture system

As mentioned above, aquaculture is one of the fastest growing sectors in the world, allowing us the maintenance of the current consumption per capita (Zhang *et al.*, 2011). This growth raises ecological concerns related to the quality and safety of products and

environment due to the large consumption of water and untreated water discharge (Rijn, 2013).

Aiming at sustainable activity, it became necessary to develop new farming methods in order to reduce the ecological impact in relation to waste production, water use and utilization of fossil fuels (Zhang *et al.*, 2011). An effective solution that emerged was the RAS (see Figure 11), where the water is partly reused after mechanical and biological treatment, to reduce water and power consumption, the release of nutrients to the environment (eutrophication), nutrient recycling, better management of health and diseases and control of biological pollution (Martins *et al.*, 2013).

This system has been adapted for cultivation of different salt and freshwater species and seafood products at facilities such as hatcheries and fish growing (Zhang *et al.*, 2011).

The main stages of the treatment of water in recirculation systems consist in the removal of solids by physical processes and ammonia conversion to nitrates by biological processes (Rijn, 2013).

Figure 11 – RAS system in Safiestela.

7.1. Removal of solid waste

The solids generated in the culture tanks (faeces and uneaten feed) are the main source of organic and inorganic waste in the system. These include dissolved and particulate organic matter (DOM and POM, respectively), and nutrients such as nitrogen (mainly inorganic) and phosphorus (Wik *et al.*, 2009).

The solids with more than 100 micron represent approximately 50% of total solids and can be easily removed from the system using settling tanks or by cleaning the cultivation tanks (Rijn, 2013).

Suspended solids (40-100 micron) make up about 25% of total solids and are suspended in the water column. These are removed by mechanical filtration, using mainly screens or sand filters (Wik *et al.*, 2009).

The dissolved solids (<40 microns) which comprise amino acids, proteins, carbohydrates, among others, are removed by foam fractionators by a process called "protein skimming". During this process, the water enters into the “skimmer”, where some air bubbles are released from below. The bubbles rise through the water column and adhere to dissolved matter leading to a foam, which can be removed. The removal of solids ensures the elimination of the non-soluble fraction of nitrogen and phosphorus (Piedrahita, 2003).

7.2. Oxidation of nitrogen compounds

The dissolved nitrogen is excreted mainly in the form of urea and ammonia, being the ammonia the main compound excreted by teleost fish. Ammonia is toxic to fish and can lead to death. Therefore it is necessary to control the ammonia levels in the tanks within safe concentrations (Rijn, 2013).

The water after solids removal goes to the biological filters. The biofilter contains a substrate (usually a plastic structure that increases the contact surface area of the bacteria with water), in which the nitrifying bacteria are fixed – bacteria of the genus *Nitrosomonas* perform the oxidation of ammonia to nitrite and bacteria of the genus *Nitrobacte*r oxidize nitrite in nitrate. These oxidation reactions represent the nitrification process (Figure 12) (Wik *et al.*, 2009). In an anoxic denitrification, facultative heterotrophic bacteria reduce nitrate to nitrogen gas (Piedrahita, 2003).

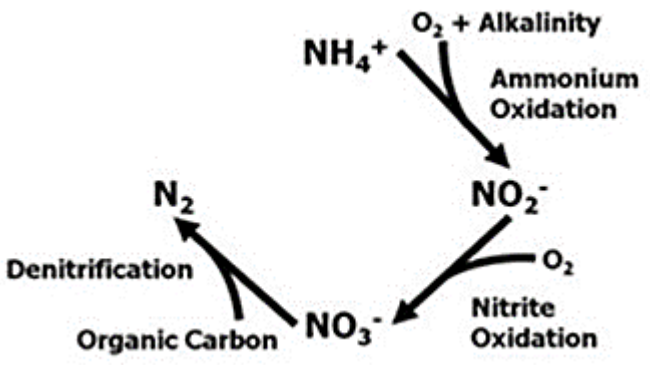

Figure 12 – 1) Oxidation of ammonia into nitrites by bacteria *Nitrosomonas,* 2) Oxidation of nitrites into nitrates by Nitrobacter. 3) anoxic denitrification. (picture from http://www.appropedia.org/Nutrient_removal).

In addition to the large oxygen consumption during the nitrification process, carbon dioxide production occurs with consequent acidification of water (due to H^+ ions). So a constant aeration of the bio-filters (Figure 13) allows to increase the concentration of oxygen and reduce the carbon dioxide (Piedrahita, 2003). The addition of limestone or hydrated lime also allow restore the pH and alkalinity of the system water (Wik *et al.*, 2009).

Figure 13 - Biofilter of the RAS system (Safiestela).

7.3. Water sterilization

In the water recirculation systems two different types of water sterilization processes can be used: the ozone and ultra-violet (UV) radiation.

The introduction of ozone in the system allows to disinfection of water by disabling pathogens, and optimize water quality by oxidizing organic waste and nitrites (Summerfelt *et al.*, 2009).

The amount of ozone supplied should be proportional to the food added, since an excessive use of ozone can lead to increased concentration of bromide in the water (by-product of ozone) and, consequently, can affect fish (Martins *et al.*, 2011).

The ultraviolet radiation uses a different technology and by radiation is capable of denaturing the DNA of microorganisms and also inactivating them (Summerfelt *et al.*, 2009).

The water should be treated to remove solid particles, before passing through the sterilization system with UV radiation to increase the efficiency of the process (Rijn, 2013).

8. A day's work in Safiestela, S.A.

Taking into account the information above mentioned, this section will briefly describe the day by day in the aquaculture.

In the company, work hours are between the 8:00 a.m. and the 5:00 p.m. (scheme of one day of work in Figure 14). Depending on the area where the intern is assigned, the procedures and care with the sole fish differ.

In the adults room, the daily work focuses on cleaning the tanks, controlling the physical-chemical parameters of the water, observation of the general condition and behavior of sole adults and on their feeding. The egg collector tanks are observed daily and if there are eggs, these are removed, numbered and placed in the incubation room to hatch. Generally, this area does not require a lot of work, so it is done in the morning, and is possible to assist other areas.

One day after hatching, the larvae density per tank is measured and the larvae are moved to the room where they are kept until larval metamorphosis. At the time of reproduction and larval maintenance, an employee is responsible for moving the larvae of the incubation room to the room where the larvae are fed with rotifers and brine shrimp several times a day during 12 days, according to the company's protocol. At this stage, it is essential to control the physical and chemical parameters of the water and the predatory behavior of the larvae (sign of predatory activity indicates good condition and quality of larvae). After metamorphosis the larvae are moved to the weaning room.

In the live food room, the larvae feed preparation starts two days earlier. Artemia eggs are placed in water 48 hours prior to the feeding of larvae. After 24 hours, artemia hatches and is filtered to remove the non-hatched cysts and empty capsules. After filtration, the newly hatched artemia is enriched for 24 hours for administration to the larvae. Rotifers are filtered on 3rd day of growth. Part of the rotifers are enriched for later administration to the larvae and another part will be used to start a new cycle. They are fed with a mixture of algae and yeast and enriched the day prior to administration to the larvae. It is necessary to control the temperature (*i.e.* dimmer) and pH (in the case of rotifers with CO_2 injection and in the case of brine shrimp with CO_2 injection and caustic soda). The emptied tanks are cleaned and disinfected, and may receive new brine shrimp eggs for hatching or rotifers for growth.

The area of weaning is the one that requires more work and care. At this stage, as highlighted earlier, the larvae are exposed to a very stressful environment due to the withdrawal of live food and introduction of inert food. It is crucial to control the water quality (e.g. measurement of physical and chemical parameters) and to avoid disturbing the larvae. Indexes of great stress may trigger outbreaks of disease and possibly death. In the weaning

room, the tanks are washed gently by removing excess organic matter and the size grading is done to minimize competitiveness between individuals and increase growth. The dead or diseased fish are removed, reducing the chance of spread of diseases. This room has about 50 tanks that are checked 2 times a day.

In the pre-ongrowing sole room, there are about 50 tanks with dimensions of 12x2x0.20 m. In the morning the maintenance and cleaning of all tanks is performed (e.g. tanks and water inlets and outlets). During this period, the dead or sick fish are also removed. It is also done an analysis of the physical and chemical parameters daily. Depending on the growth of the tanks, it is necessary to make a size grading of the sole fish. Since fish are larger and more numerous, the company uses in this operation an automatic calibrator that generally requires three persons. One of them collect the sole in the tank, the second receive the fish and places them gently in the calibrator and the other one removes the fish calibrated to place them in the new tank taking into account the size. The emptied tanks require a cleaning and disinfection before receiving new fish. In the afternoon, the general conditions of the fish and the tanks are observed and, if necessary, the size grading continues. If there is no fish to transfer or calibrate, usually the time is devoted to the room cleaning and disinfection. Once a month, the larger fishes are transported by truck from Safiestela to Aquacria, where the sole will grow, until the size suitable for sale is reached.

The feed of the fish of the weaning and pre-ongrowing room is done by automatic feeders controlled by a central computer. Therefore, it is necessary to calculate the fish average weight weekly, in order to adjust the amount and size of food delivered by the automatic feeder.

The maintenance of the machines attached to the tank (e.g. air pumps, water pumps, automatic feeders) and filtration system (*e.g.* RAS) is made by specialists in order to maintain the performance of the equipment.

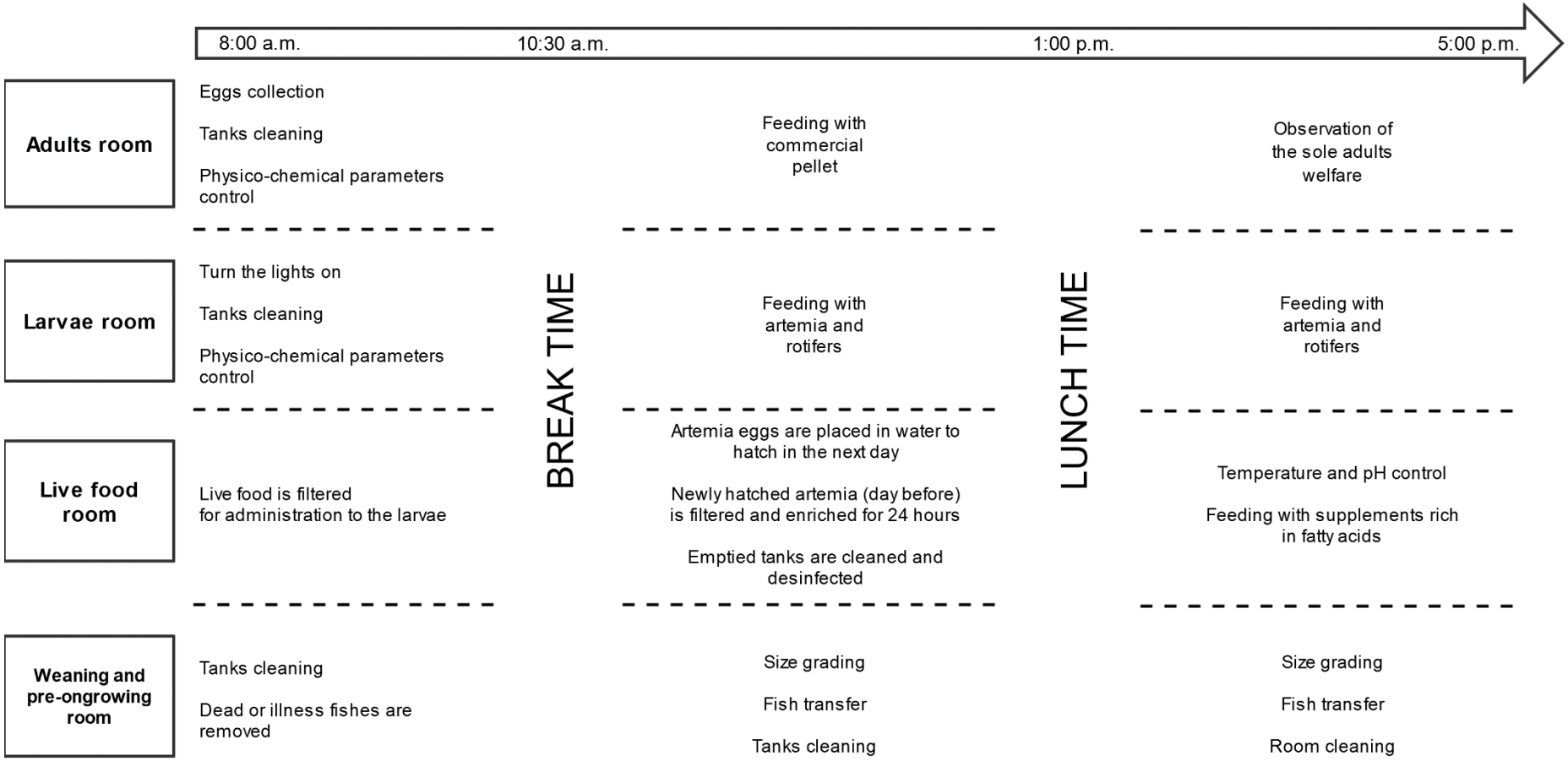

Figure 14 - Illustrative scheme of one day of work on Safiestela company.

III. Evaluation of the *Pediococcus acidilactici* bacteria strain effects on growth performance of *Solea senegalensis*

Senegalese sole is a promising flatfish species for intensive farming due to its high market value and demand in Europe, as aforementioned. Notwithstanding recent developments in rearing management and feeding techniques (Lobo *et al.*, 2014b), the production of sole still has some problems (Dâmaso-Rodrigues *et al.*, 2010) such as difficulties to control diseases, fulfill larval and juvenile nutrient requirements in captivity and in the optimization of feeding and husbandry protocols (Morais *et al.*, 2014a), as well as reproduction of G1 generations. In order to obtain a high quality larvae and juvenile, proper nutrition at first feeding is of extreme relevance (Dâmaso-Rodrigues *et al.*, 2010).

The intensive production involves subjecting animals to different stressor agents (management, density, among others) that may weaken their immune system and allow the action of opportunistic pathogens (Tapia-Paniagua *et al.*, 2012). The Table 8 summarizes the principal sole diseases in intensive aquaculture.

The gastrointestinal tract is a route for access of pathogenic organisms, as *Photobacterium damselae piscicide subs*, *Vibrio harveyi* and some *Tenacibaculum* species, which has raised obstacles in the production of this flat fish species (Piñeiro-Vidal *et al.*, 2008a; Piñeiro-Vidal *et al.*, 2008b). There is a vaccine for sole against *Photobacterium damselae piscicide* and *Vibrio harveyi subs*, but only a temporary protection is achieved (Arijo *et al.*, 2005). There are also viral diseases like *Betanodaviruses* (Hodneland *et al.*, 2011), *Birnavirus* and *Lymphocystis virus* (Cano *et al.*, 2010) that were detected in sole culture.

Tenacibaculosis (primarily caused by *Tenacibaculum maritimum*) induces morbidity and mortality in aquacultures in several countries, affecting the cultivation of marine fish species. Affected sole show external signs as eroded mouth, corroded fins and skin lesions (Morais *et al.*, 2014a).

Photobacteriosis caused by *Photobacterium damsela spp. Piscicide*, is responsible for heavy losses, causing high mortality. Juveniles are mainly affected with acute mortality, but they do not exhibit signs of external injuries. This disease affects farmed sole above 18ºC (Magariños *et al.*, 2003). Vibrio infections are generally considered a secondary infection, being the presence of ulcers on the skin and bleeding areas on the fins and mouth the main external symptoms observed (Zorrilla *et al.*, 2003).

However, recent studies have shown positive results in the use of probiotics to control different species of *Vibrio and Photobacteriosis* (Díaz-Rosales *et al.*, 2009; García de la Banda *et al.*, 2012; Tapia-Paniagua *et al.*, 2012).

Table 8 - Diseases reported in Senegalese sole culture, adapted from Tapia-Paniagua *et al.* (2012) and Morais *et al.* (2014a).

		Pathogenic organism	Reference
Bacterial diseases	**Pseudotuberculosis**	*Photobacterium damselae subsp. piscicida*	(Zorrila *et al.*, 1999)
	Vibriosis	*Vibrio harveyi* and *V. parahaemolyticus*	(Zorrila *et al.*, 2003)
	Tenacibacteriosis	*Tenacibaculum maritimus* and other *Tenacibaculum* species	(Pineiro-Vidal *et al*., 2008)
Viral diseases	**Lymphocystis**	*Lymphocystis vírus* and others as *Birnaviruses* and *Betanodaviruses*	(Cano *et al*., 2010)

In addition to the described diseases, deformities in the skeleton and pigmentation problems are also factors affecting the productivity and income of sole culture (Boglione *et al.*, 2013a; Boglione *et al.*, 2013b). Fish with pigmentary disorders cannot be marketed because they do not fit in the quality standards (Darias *et al.*, 2013a; Darias *et al.*, 2013b).

The skeletal deformities usually arise due to genetic factors or the presence of a stressful environment that affects the growth and animal movement (Boglione *et al.*, 2013a), being the larval nutrition one of the main parameters affecting the skeletogenesis during the larval development (Morais *et al.*, 2014a).

Pigmentation abnormalities are featured by a deficiency in pigmented cells in the eye side or excessive pigmentation on the blind side (Bolker and Hill, 2000). These abnormalities may be a consequence of ARA (Arachidonic acid) levels in the diet (Boglino *et al.*, 2014), the color used in the production tanks or the intensity of light used. An elevated light intensity and a transparent or light colored tank may affect the larvae pigment (Lund *et al.*, 2010).

These factors, that affect the growth performance, can be overcome with supplementation with probiotics. There are studies that prove that these reduce the incidence of diseases, improve the general condition and welfare of animals, provide

nutritional advantages and modulate the immune system, making larvae more resistant to stress environments, promoting a decrease in the incidence of skeletal deformities and depigmentation (Mohideen *et al.*, 2010; Wang and Gu, 2010).

Initial studies used probiotics during juvenile and adult stage, but lately the probiotics have begun to be used in the early stages of life of aquatic animals (Vine *et al.*, 2006). When the marine fish larvae hatch have an undeveloped digestive system. During development this system can be colonized by the egg microbiota, by surrounding bacteria present in the water or also in the beginning of the feeding (Lobo *et al.*, 2014b).

Larvae have a poorly developed immune system (Vine *et al.*, 2006) and the colonization of the gut and environment by probiotics may decrease the exposure of larvae to pathogenic bacterial agents and can provide a gut balanced microbiota condition (Tinh *et al.*, 2008).

Bactocell PA is the trade name for a feed additive based on viable cells of a lactic acid bacteria *Pediococcus acidilactici.* This product is already licensed for several species of fish. It is used in fish, in order to improve the quality of the animal product by increasing the number of well-conformed fish (fish with less structural deformities), increase the weight gain, reduce the incidence of deformities and diseases, as shown in studies with seabass, trout and salmon (EFSA, 2009).

Considering the information mentioned above, the main aim of this study was the evaluation of the effect of a brief probiotic administration during larval phase (2-12 DAH) on growth performance and gut microbiota colonization in *Solea senegalensis* larvae.

1. Methodology

1.1. Microorganisms

Bactocell PA is stored as a fine white powder with the concentration of *Pediococcus acidilactici* MA18/5M of $1x10^{11}$ CFU/g, according to the European Food Safety Authority (EFSA, 2009).

In the first assay, *Pediococcus acidilactici* cells were administered directly in the tank water and bioencapsulated (using artemia and rotifers) in one of the groups. In the other group the probiotic was given only via live food, using artemia and rotifers as vectors.

In the second assay, probiotic cells were supplied directly in the rearing water in the morning, with the concentration recommended by the brand ($1g/m^3$/day). Probiotic was previously diluted in 500 mL of salt water and then homogeneously distributed in the tank.

1.2. Larval rearing conditions

Embryos were obtained from natural spawning of wild Senegalese sole broodstock kept in Safiestela (Estela, Póvoa Varzim, Portugal).

In assay one, eggs were incubated at 19ºC in 100 L cylinder-conical incubating tanks with gentle aeration and a continuous water flow. Newly hatched larvae were distributed into 25 L circular tanks in triplicate, with constant aeration and water renewal (density of 16 larvae per liter). This trial lasted 12 days and the larvae were always kept in the same tanks and conditions. Larvae were discarded after metamorphosis.

During the trial, the temperature was maintained between 18.8 and 19.4ºC and salinity at 35 g/L. Light intensity, between 650 and 1000 lux at the surface, was provided by halogen lamps, with 16L:8D cycle. After metamorphosis, post larvae were reared in semidarkness (200 lux at surface).

Three feeding regimes (three replicates each) were compared in the first assay: one of the probiotic group received the bactocell bacterial strain using rotifers and artemia as living vector from 3 to 12th DAH (rotifers and artemia were enriched with commercial probiotic during the night; 2.5 g/m^3/day were added in rotifers tank and 5 g/m^3/day were added in artemia tank, according to manufacturer's instructions) and the other received the probiotic via live food and directly at the rearing water (1 g/m^3/day were added in the rearing water, according to manufacturer's instructions). In both probiotic treatments the bacterial strain were given one time a day in the morning, whereas no bacteria were administered to the control group.

In the second assay, embryos were incubated at 19 ºC in 100 L in the same conditions as assay 1. Newly hatched larvae were distributed into 2,800 L circular tanks (industrial conditions) in duplicates, with constant aeration and water renewal (density of 28 larvae per liter).

Temperature varied between 18.9 to 19.2 ºC between the 2-12th DAH and 18.7 to 20.1 ºC between 12-45th DAH. Salinity was 35 g/L throughout the trial, while illumination, around 1000 lux at surface, was provided by halogen lamps, with 16L:8D cycle between the 2-12th DAH. Continuous water flow was maintained and oxygen and N-compounds were suitable for the larvae and post-larvae culture (Morais *et al.*, 2014a).

After metamorphosis, the larvae became benthic and the post larvae were distributed in relation to both treatments in other circular tanks with 20 cm depth (density of 3,000 larvae per m^2), until the end of the trial (Figure 15). Post larvae were reared in semidarkness (200 lux at surface). In these tanks, the conditions were similar to those used in the previous ones.

Two feeding regimes (with two replicates each) were compared in the second assay: bactocell and control groups. Bactocell group received the *Pediococcus acidilactici* bacterial strain homogeneously distributed in the tank (1 g/m^3/day were added in the rearing water). The probiotic was given one time a day from 2 to 12th DAH, whereas no bacteria were administered to the control group. After 12 DAH and until the weaning, all larvae were fed with live artemia. Each treatment was made in duplicate (Figure 15).

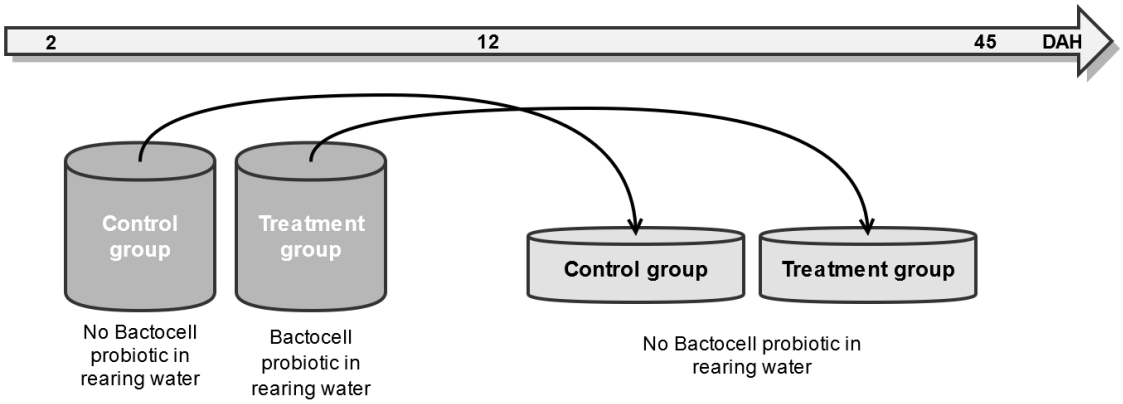

Figure 15 - Schematic of the probiotic feeding regime for the second trial, based on the protocol described in the text. Each treatment was made in duplicate.

The feeding regime was followed according to the protocol described by Imsland *et al.* (2003) for both assays (Table 9). From 3 to 8th DAH enriched rotifers were added to the tanks 4 times a day to maintain the rotifer density of 2 rotifers per mL. During this period, *Nannochloropsis sp* (3x10^5 cel/mL) were also added in the rearing tanks (around 30 mL of microalgae concentration in 3000 liters of water) to ensure a good rotifer quality and increase contrast improving larvae capacity to detect the prey. Rotifers were enriched with a mixture of yeast and microalgae. From 5 to 9th DAH newly hatched artemia (AF strain INVE Aquaculture, Ghent, Belgium) were added to the tanks 4 times a day in order to maintain the artemia density of 0.5 artemia per mL. From 8 to 31th DAH enriched metanauplius of artemia (EG strain INVE Aquaculture, Ghent, Belgium) were added to the tanks 4 times a day to maintain the artemia density of 1.5-5 artemia per mL. Artemia EG strain were enriched with DHA Super Selco (INVE Aquaculture, Ghent Belgium) for 18 hours. Finally, from the 31th DAH until the end of the trial (45 DAH), the feeding was carried out with commercial pellet Gemma Diamond (crude protein 60% and total lipids 15%, Skretting, Burgos, Spain) eight times a day. The amount of inert feed was gradually increased, accordingly the mean weight of the rearing tank, weighted weekly. After 45 DAH, the sole individuals of the experiment have continued the normal production cycle of the company.

Table 9 – Sole feed regimes timeline (Imsland *et al.*, 2003).

	DAH			
	3-8	**5-9**	**8-31**	**31-45**
Rotifers				
Artemia AF strain				
Artemia EG strain				
Commercial pellet Gemma Diamond				

1.3. Growth parameters

In the first assay the density was 400 larvae per tank, while in the second assay was 3,500 larvae per m^2.

For growth studies, 3 specimens from each replicate were randomly sampled each day between 2-11 DAH and 20 specimens from each replicate in 12 DAH in the first assay. Additionally, for the larvae dry weight analysis, 20 larvae per replicate were sampled in 7 DAH and 40 individuals per replicate in 12 DAH.

In the second assay, 10 specimens were randomly sampled from each replicate every day between 2-11 DAH and 30 specimens were sampled from each replicate in 12, 20, 25, 31 and 45th DAH. Fish total length was measured using a micrometer in a microscope in both experiments.

After length measurement in the 3,12, 20, 25, 31 and 45 DAH, larvae were rinsed with distilled water, stored into eppendorf tubes and dried at 60 ºC for 48 h, in order to obtain larval dry weight. The same fish were used for total length and dry weight analysis.

The growth performance were assessed using the following parameters:

- Condition index (K) was calculated using the following formula: K= FBW (g)/[length (cm)]^3 x 100, where FBW is the final body weight (Dinis *et al.*, 2007).
- The relative growth rate (G) was determined using the formula $G=100(\ln S_2-\ln S_1)(t_2-t_1)^{-1}$, where S_1 and S_2 are initial and final mean total length respectively in mm, and t_1 and t_2 are the days of measure (Forsythe and Van Heukelen, 1987).
- Specific growth rate (SGR). SGR=100((lnFBW-lnIBW)/T), where FW is the final body weight (g), IW the initial body weight (g) and T is the duration of feeding in days (Ferguson *et al.*, 2010).

1.4. Larval metamorphosis index (larval stage)

According to Fernández-Díaz *et al.* (2001), there are 5 larval stages during the development of the sole larvae. The following table describes the specifications for each stage.

Table 10 – Larval stages of sole larvae, according to Fernández-Díaz *et al.* (2001).

Stage	Description
0	Symmetric larvae with vertical swimming plane (pelagic)
1	The left eye starts to migrate toward the dorsal position
2	The migrating eye can be seen from the right ocular side
3	The individuals change their swimming plane (horizontal; benthic) and the eye continue the migration in the ocular side
4	Eye translocation is finished and the orbital arch is visible

For the study related to the larval metamorphosis index, 100 larvae were sampled from different control groups and 20 from probiotic groups since 2 to 15th DAH. The aim of this study was to determine which days predominate the different stages and, also, the proportion of larvae in stage 3 in 12-13 DAH and stage 4 in 14-15 DAH.

The eye migration index was calculated using the formula: IEM = Σ (% fish in each stage * stage) / 100 (Solbakken *et al.*, 1999).

1.5. Gut microbiota

In the same way as the previous topics, for the evaluation of the gut microbiota composition four samples were randomly obtained from each replicate on 12 DAH. Fish samples were washed with distilled water, placed into eppendorf tubes and then stored at -20 ºC, until analysis. This procedure aims the comparison of Denaturing Gradient Gel Electrophoresis (DGGE) patterns of the intestinal microbiota of soles receiving different experimental treatments.

1.5.1. DNA extraction

The PCR-ready genomic DNA were isolated from the sole samples using the FastDNA® SPIN kit (MP Biomedicals) following the manufacturer's instructions. Briefly, the whole gut of 20 fish per sample was aseptically removed with the assistance of a stereomicroscope and keep overnight in ethanol PA 70% at -20 ºC into the Lysing Matrix E tubes containing a mixture of ceramic and silica particles until further analysis. The intestinal

contents were individually homogenized, centrifuging each one at 14,000 rpm for 5 min. The microbial cell lysis was performed on the FastPrep® Instrument (Q Biogene) for 40 s at the recommended speed. The extracted DNA was eluted into DNase/Pyrogen-Free Water and stored at -20ºC until use (with some modifications as described by Polónia *et al.* (2014)).

1.5.2. Nested PCR and DGGE

In order to compare Denaturing Gradient Gel Electrophoresis (DGGE) patterns of the intestinal microbiota of soles receiving the different diets, a nested PCR technique was used to amplify the 16S rRNA gene fragments from the total bacterial community DNA suitable for bacterial DGGE fingerprints (Gomes *et al.*, 2008).

In the first PCR (24 cycles), the bacterial primers F-27 and R-1492 were used to amplify approximately 1,450 bp of the 16S rRNA gene (Weisburg *et al.*, 1991). The amplicons obtained from the first PCR were then used as a template in a second PCR (24 cycles) with bacterial DGGE primers F984-GC and R1378 (approximately 473 bp) (Heuer *et al.*, 1997). Bacterial DGGE was performed on a DCode universal mutation detection system (Bio-Rad, Hercules, CA) with a denaturing gradient of 40 to 58% (100% denaturant contains 7 M urea and 40% formamide) (Martins *et al.*, 2013). Amplicon separation was achieved in 1X TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) at 58 ºC for 16 h at a constant voltage (70 V). The gel was silver stained according to the method of Heuer *et al.* (1997).

1.6. Statistical analysis

All data corresponding to growth are presented as mean ± standard deviation (SD). The coefficient of variation (CV) was determined with the formula: (treatment standard deviation/treatment mean) x 100 (Sokal and Rohlf, 1981), to determine inter-individual length variation within the same treatment.

A one way ANOVA was performed to detect statistically significant differences in growth between treatments, after testing the necessary assumptions (normality with Kolmogorov-Smirnof test and graphical criteria and homogeneity of variances with Levene's test). All statistical analyses were performed using SPSS v23 software with two-sided significance set at 5% throughout.

The gel image of the DGGE was acquired with an Epson Perfection V700 photo scanner, and the digitalized profiles were analyzed with the software package GelCompar 4.0 (Applied Maths, Austin, TX) following the method of Gomes *et al.* (2010). The DGGE

band surface was converted to relative intensity by dividing its surface by the sum of all band surfaces in a lane. This value was then log (x + 1) transformed, and a distance matrix was constructed using the Bray-Curtis index in PRIMER 6 (Clarke and Gorley, 2001). Variation in bacterial composition among fish groups was assessed with multidimensional scaling analysis in PRIMER. It was tested for significant differences in the gut bacterial community among fish groups using an ANOSIM analysis in PRIMER with 999 permutations. The R statistic in ANOSIM ranges from 0 to 1. In general, R > 0.75 indicates strong separation, R > 0.5 and < 0.75 indicates moderate separation, and R < 0.25 indicates poor separation (Pegoraro *et al.*, 2015).

2. Results

In this section the results obtained from the experiment performed at Satiestela, SA will be discussed. This project intended to assess the influence of the *Pediococcus acidilactici* bacterial strain (Bactocell) in the growth of *Solea senegalensis* species. In order to fulfil this purpose, two main trials were performed: one in a laboratory scale, and a second at an industrial scale.

In the first trial, to evaluate the influence of the Bactocell probiotic into the *Solea senegalensis* larvae growth, three independent conditions were tested: (1) no bacteria administered (control group); (2) insertion of the Bactocell bacterial strain via live food and directly in the rearing water; and (3) insertion of the Bactocell bacterial strain only via live food from 3 to 12th DAH. The aim of this trial was to analyze if this probiotic could positively interfere with the larvae growth over time.

According to Figure 16 and the statistical analysis performed (data not shown), there were no significative differences ($p>0.05$) between the total length of the sole larvae for the three different feed regimes. This indicates that, apparently, Bactocell bacterial strain did not influence larvae length in the period.

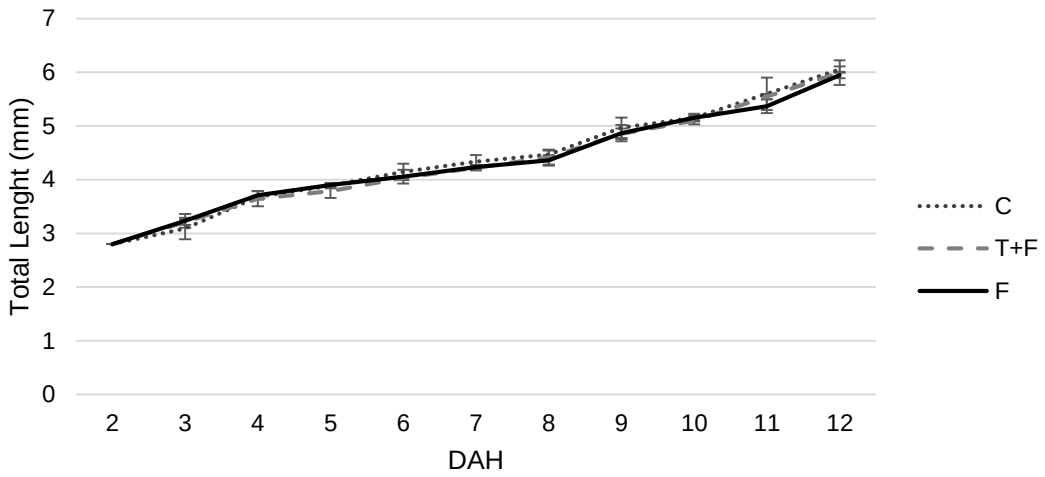

Figure 16 – Temporal changes in the total length (mm) of the Senegalese sole larvae under study along the different days after hatching (2-12 DAH) in the first trial. No significant differences were observed in the different feed regimes applied. Data are expressed as mean ± SD (n=3). C: Control (absence of probiotics in the tank and in the feed); T+F: probiotics inside the tank and also in the feed; F: probiotics only in the feed.

At the same time, Figure 17 represents the variation of the larvae dry weight over the period. In the first trial, the dry weight was only measured in the 3rd, 7th and 12th DAH. These results also demonstrate that there were not statistically significant differences in the dry weight of the larvae between the different feed regimes (with or without probiotic) tested and in all DAH analyzed ($p>0.05$).

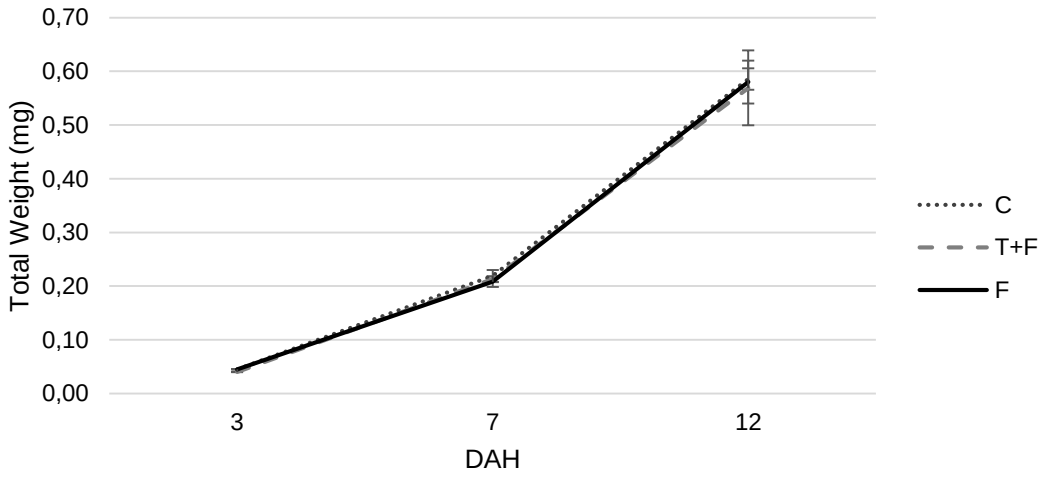

Figure 17 –Temporal changes in the dry weight (mg) of the Solea senegalensis larvae along the different days after hatching (2-12 DAH) in the first trial. No significant differences were observed in the different feed regimes applied. Data are expressed as mean ± SD (n=3). C: Control (absence of probiotics in the tank and in the feed); T+F: probiotics inside the tank and also in the feed; F: probiotics only in the feed.

The initial length of the larvae were the same for each condition (2.80±0.00 mm, see Table 11), this could be due to all sole larvae used in the trial were obtained from the same hatching tank and from the same spawning. However, independently of the absence or presence of Bactocell in the tank and/or in the feed, there were not any significative differences in the final length, daily growth rate, initial and final weight and also in the weight gain, as represented in the Table 11 ($p>0.05$).

Table 11 – Growth performance of Senegalense sole larvae in the three feed regime groups. Data are expressed as mean ± SD (n=3). No significant differences were observed in the different feed regimes applied. C: Control (absence of probiotics in the tank and in the feed); T+F: probiotics inside the tank and also in the feed; F: probiotics only in the feed.

Parameters	C	T+F	F
Initial length (mm)	2.80±0.00	2.80±0.00	2.80±0.00
Final length (mm)	6.05±0.06	6.00±0.23	5.95±00.06
Relative growth rate (% day $^{-1}$)	7.71±0.1	7.61±0.38	7.54±0.09
Initial weight (mg)	0.05±0.00	0.04±0.00	0.05±0.00
Final weight (mg)	0.59±0.02	0.57±0.07	0.58±0.04
Weight gain (mg/fish/day)	0.06±0.00	0.06±0.01	0.06±0.01
Specific growth rate (% day $^{-1}$)	27.35±0.44	29.46±1.37	27.08±0.92

As a conclusion of this preliminary trial, apparently the Bactocell probiotic did not influenced the growth of the sole larvae. For this reason, a second trial was performed on an industrial scale. Unlike the first trial, only two conditions were tested to evaluate the potential effects on the sole larvae growth when fed with *Pediococcus acidilactici* bacterial strain: (1) a control group where no bacteria was administered in the feed regime and (2) a treatment group where the probiotic under evaluation was only added to the rearing water in the culture tank. In this trial, it was decided not to test the enrichment of Bactocell probiotic in the live prey, since it was not known if this type of enrichment could affect fatty acid composition of the live prey, therefore negatively influencing growth and deformation index. For future development a preliminary trial on the effect of Bactocell in the fatty acid profile of the live prey need to be performed.

Concerning the second trial, it was analysed the total length of the larvae during over the days after hatching (Figure 18). As expected, the larvae length increased during the trial for both groups. The total length of the larvae of the treatment group was clearly greater than the control since the 25th until the 45th DAH. These data suggest that Bactocell starts to influence the larvae growth since the 25th DAH. There were significant differences ($p<0.05$) in the total length at the 7th (4.70±0.04 and 4.89±0.05 mm in the control and

treatment groups, respectively), 9th (5.62±0.02 and 5.71±0.01 mm in the control and treatment groups, respectively) and 31th DAH (4.70±0.04 and 4.89±0.05 mm in the control and treatment groups, respectively). Despite these results, more studies are needed to understand the effect of Bactocell in the growth of sole larvae in longer periods of evaluation.

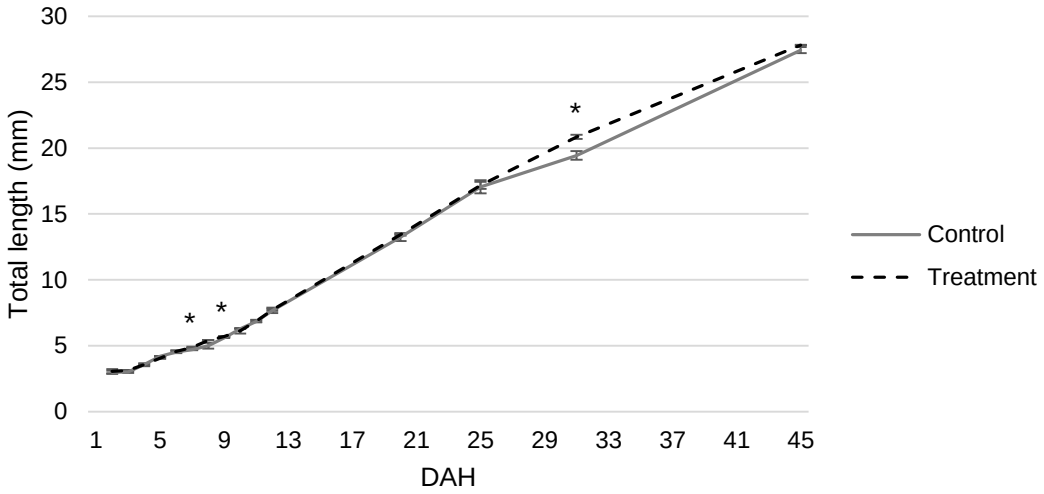

Figure 18 - Temporal changes in the total length (mm) of the Senegalense sole larvae along the different days after hatching (2-45 DAH) in the second trial. At the 7, 9 and 31th DAH it was observed significant differences between the control and the treatment group. Values are mean ± SD of duplicate determination. Asterisk (*) denotes significant difference regarding the comparison between treatments in the same sampling days (P<0.05). Control: absence of probiotics in the tank and in the feed; Treatment: probiotics inserted inside the tank.

For the evaluation of dry weight results along the period, no significant differences were observed between the control and treatment group (p>0.05 for all DAH). In fact, the graph in Figure 19 demonstrates a complete overlap of both curves, suggesting that the use of *Pediococcus acidilactici* bacterial strain did not influence the dry weight of the sole larvae, contrary to what was observed in the total length after 25 DAH.

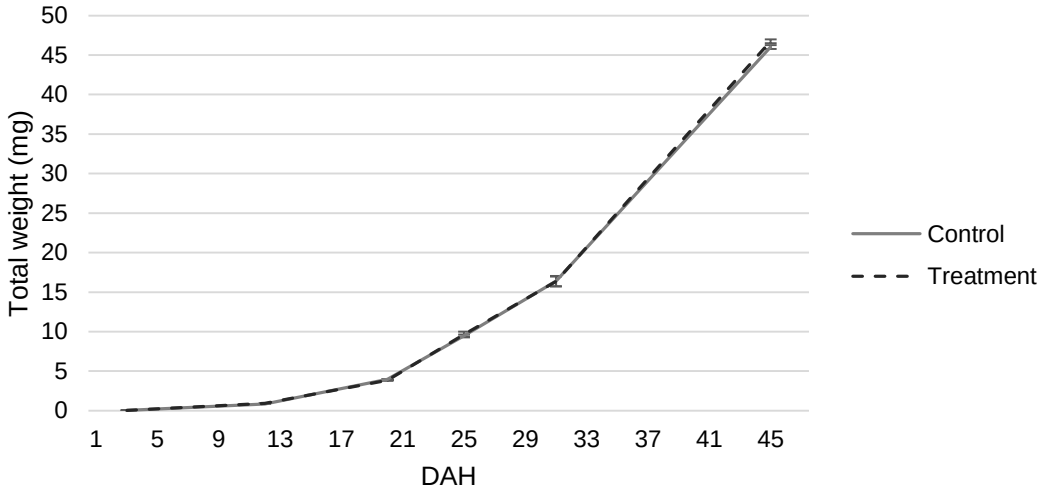

Figure 19 - Temporal changes in the dry weight (mg) of the Solea senegalensis larvae along the different days after hatching (2-45 DAH) in the second trial. No significant differences were observed in the different feed regimes applied. Values are mean ± SD of duplicate determination. Control: absence of probiotics in the tank and in the feed; Treatment: probiotics inserted inside the tank.

Average of total length and weight at the end of the period were similar in Bactocell (27.82±0.03 mm) and the control group (27.44±0.23 mm), as can be seen in Table 12.

In contrast to the first trial, the sole larvae used were hatched in different tanks and were from different spawning, leading to different initial length for both conditions (2.98±0.11 and 3.06±0.16 mm for the control and treatment group respectively). Similarly to the preliminary trial, there were no significative differences in the final length, diary growth rate, initial and final weight between the two groups ($p>0.05$), as observed in Table 12.

Table 12 - Growth performance of *Solea senegalesensis* larvae in both control and treatment groups. Values are mean ± SD of duplicate determination. Control: absence of probiotics in the tank and in the feed; Treatment: probiotics inserted inside the tank.

Parameters	Control	Treatment
Initial length (mm)	2.98±0.11	3.06±0.16
Final length (mm)	27.44±0.23	27.82±0.03
Relative growth rate (% day $^{-1}$)	5.16±0.02	5.13±0.00
Initial weight (mg)	0.04±0.00	0.04±0.00
Final weight (mg)	46.02±0.25	46.75±0.24
Weight gain (mg/fish/day)	1.09±0.01	1.11±0.00
Specific growth rate (% day $^{-1}$)	16.78±0.01	16.82±0.01
Survival rate (%)	92%	94%

The condition index was calculated as described previously by Dinis *et al.*, 2007. This parameter allows the characterization of the fish species, such as sole, in relation to the weight and length, and it should be superior to 1. The values obtained for each group were very similar for the different DAH (since the 12-45 DAH) (Table 13), which illustrates that, apparently, the treatment with Bactocell probiotic in the tank did not influence the condition index of the *Solea Senegalensis* larvae (statically there are no differences, $p>0.05$).

Table 13 – Condition index of the control and treatment groups since the 12 to 45th days after hatching (DAH). Data are expressed as mean ± SD (n=2). Control: absence of probiotics in the tank and in the feed; Treatment: probiotics inserted inside the tank.

DAH	Control	Treatment
12	1.89±0.02	2.01±0.15
20	1.76±0.01	1.72±0.15
25	1.93±0.02	1.86±0.01
31	1.99±0.04	2.12±0.00
45	2.23±0.05	2.22±0.10

The use of *Pediococcus acidilactici* probiotic diminished the sole growth heterogeneity (Table 14), with statistically significant differences ($p<0.05$) in 20 and 45 DAH. A less heterogeneous fish size in length was detected in the probiotic group (CV=4.45±0.17) compared to control fish (CV=7.97±0.03) at the end of the trial. This tendency was maintained during the experiment.

Table 14- Length dispersion of the control and treatment groups on 3rd and 12 to 45th days after hatching (DAH). To calculate length dispersion it was used the coefficient of variation (CV). Data are expressed as mean ± SD (n=2). Different letters (a: control; b: treatment) denote significant differences among treatments ($P<0.05$). Control: absence of probiotics in the tank and in the feed; Treatment: probiotics inserted inside the tank.

DAH	Control	Treatment
3	3.81±0.23	5.16±0.30
12	2.90±0.06	2.67±0.73
20	4.08±0.09^{a}	2.36±0.13^{b}
25	4.73±0.69	4.21±1.14
31	6.21±1.56	4.66±0.72
45	7.97±0.03^{a}	4.45±0.17^{b}

It has been evaluated the metamorphosis larval index in order to determine in which days predominate the different stages and, also, the proportion of larvae in the different stages along the days.

In both groups the process of metamorphosis started at 7 DAH. As can be seen in the Figure 20, in 10 DAH, the treatment group had a higher prevalence of animals in stage 1 and 2 compared with control group (70% and 55% of larvae in stage 1 and 20% and 14% in stage 2 in the treatment and control group respectively).

In 12 DAH, 92% of larvae in the control group were in stage 3, while in the treatment group 100% were in stage 3. In both groups all larvae were in stage 4 (complete metamorphosis) at 15 DAH.

The comparison of IEM between groups demonstrated that there were no significative differences.

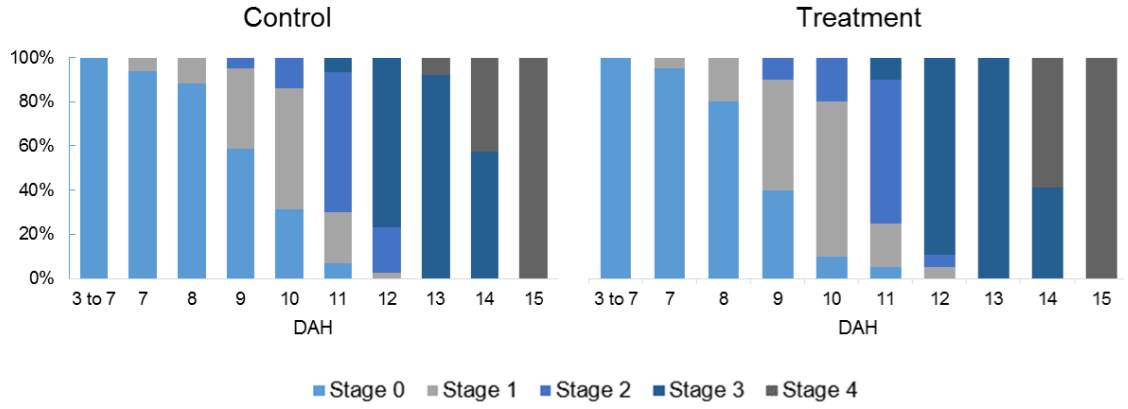

Figure 20 - Distribution of Senegalense sole individuals in the different stages during metamorphosis (%), according to the different days after hatching (DAH) of the control and treatment groups from the second assay. Data are expressed as mean ± SD (n=2). Control: absence of probiotics in the tank and in the feed; Treatment: probiotics inserted inside the tank.

The DGGE profiles bacterial assemblages of the two treatment groups in trial 2 demonstrated the dominance of different bacterial populations, as can be seen by the distinct bands in Figure 21. The results showed that the administration of *Pediococcus acidilactici* in the rearing water produced changes in the DGGE patterns of fish fed the probiotic diet in comparison with those receiving the control diet. This variability was also indicated by the multidimensional scaling ordination (see Figure 22) where it is demonstrated that the two groups (control and treatment) had a tendency to form different clusters.

The ANOSIM analysis of bacterial profiles of the two groups produced a higher R value equal to 0.699.

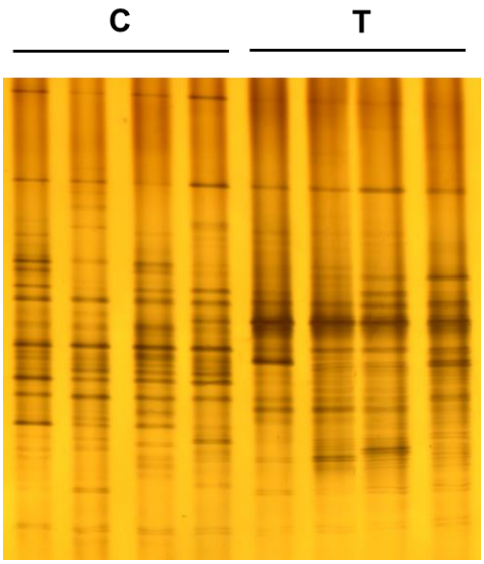

Figure 21 – Denaturing gradient gel electrophoresis (DGGE) fingerprint of 16S rRNA gene fragments amplified from four replicates of two different feed regime groups in 20 DAH. C: absence of probiotics in the tank and in the feed; T: probiotics administered inside the tank.

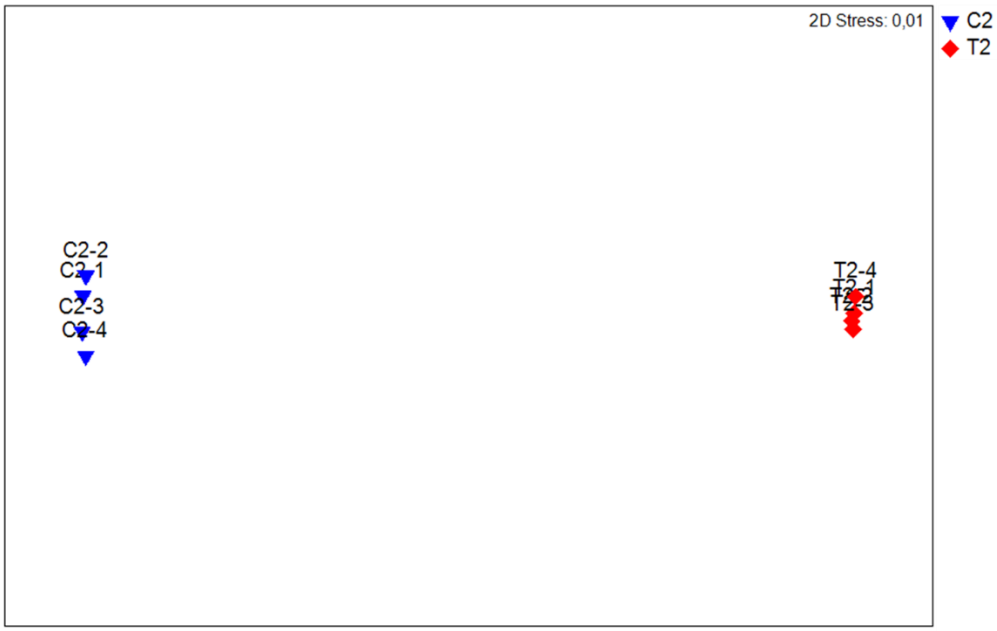

Figure 22 – Multidimensional scaling analysis of the bacterial community structure based on DGGE profiles comparing similarities between the gut microbiota in both groups in 20 DAH. Control group (∇ C2) and Treatment group (◊ T2).

3. Discussion

Lactic acid bacteria (LAB), such as *Lactobacillus spp., Carnobacterium spp. and Enterococcus faecium* have been tested as probiotics for improvement of growth in fish larvae (Ringø and Gatesoupe, 1998; Lobo *et al.*, 2014a). However, there are still gaps in the literature of some LAB, for instance, the *Pediococcus acidilactici*. This bacterium produces bacteriocins (Anastasiadou *et al.*, 2008) and organic acids (such as lactic acid and acetic acid) that have antagonistic properties against various Gram positive and Gram negative bacteria, especially *Vibrio spp.* (Beaulieu *et al.*, 2006; Castex *et al.*, 2009).There are studies that evaluate the potential of this bacteria in fish species such as pollock *Pollachius pollachius* (Gatesoupe, 2002), rainbow trout *Oncorhynchus mykiss* (Aubin *et al.*, 2005), channel catfish *Ictalurus punctat*us (Shelby *et al.*, 2007) and Nile tilapia *Oreochromis niloticus* (Shelby *et al.*, 2006). *Lactobacillus* strains have been demonstrated that can promote a better performance in larval and juvenile body weight in *Sparus aurata* (Avella *et al.*, 2010a) and in *Dicentrarchus labrax* (Abelli *et al.*, 2009).

Pediococcus acidilactici strain MA 18/5M is a LAB used to improve the gut microbiota, pathogens control and growth in different fishes as salmon, trout and tilapia (EFSA, 2009).

Improving larvae development is an important characteristic of probiotics used in aquaculture (Avella *et al.*, 2010b). Until today, several studies with probiotics shown that they provide both nutritional and protective benefits (Balcázar *et al.*, 2006; Merrifield *et al.*, 2010a). In the present work, it was observed a better growth performance of the Senegalense sole when they ingest the Bactocell probiotic, compared with the control group. However, it was only verified a statistical difference between groups in the 7^{th} ($p=0.048$), 9^{th} ($p=0.034$) and 31^{th} DAH ($p=0.027$) (Fig. 18).

Some previous studies that evaluated the effect of *Pediococcus acidilactici* on growth performance of aquatic animals have demonstrated contradictory results (Zhou *et al.*, 2010; Merrifield *et al.*, 2010a). The use of *P. acidilactici* in rainbow trout (Aubin *et al.*, 2005; Merrifield *et al.*, 2010a), Nile tilapia (Shelby *et al.*, 2006) and catfish (Shelby *et al.*, 2007) failed to improve growth performance. Despite the absence of benefits relating growth performance, this probiotic has positive effects in other areas in this species (for example, immune system, digestion, pigmentation, skeletal deformities). Although it was reported an improvement in pollock larvae weight gain when artemia enriched with Bactocel was administered and in tilapia growth performance when exposed to a different probiotic via the rearing water (Zhou *et al.*, 2010). Different results using Bactocell exhibited an increased feed conversion rates and better health and safety (Feed Mix, 2005). In aquaculture, Bactocell also controls the environment microbiota (*i.e.* tanks and ponds) and shown

different positive consequences in survival rates and performance in shrimps, salmonids and other aquatic species (Feed Mix, 2005). In salmonids, the use of Bactocell probiotic diminished the presence of vertebral column compression syndrome (VCCS) and, consequently, improved the survival rates and growth (Feed Mix, 2005).

In trout, despite not beneficial growth performance, *P. acidilactici* presented benefit results related to K-factor (condition index), leukocyte levels, colonization of the gut and the reduction of the vertebral column compression syndrome (Aubin *et al.*, 2005). Another study in *Orcorhynchus mykiss* has shown the reduction of the VCCS, using Bactocell as a food additive and in *Dicentrarchus labrax*, the use of Bactocell has diminished the incidence of bone deformation and improved mineralization (EFSA, 2009).

In shrimp *Litopenaeus stylirostris*, Bactocell demonstrated positive results regarding antioxidant defenses and oxidative stress, weight gain, survival, feed conversion ratio (Castex *et al.*, 2009; Merrifield *et al.*, 2010a).

The concentration outlined in the use of Bactocell was obtained in studies on fish as seabass and rainbow trout (EFSA, 2009), so it is necessary to optimize the dose and frequency of administration in the specie *Solea senegalensis*, in order to maximize their benefits.

Metamorphosis involves a transformation from a pelagic to a benthic position, being this process influenced by the dietary (Fernández-Díaz *et al.*, 2001). So, Bactocell appears to synchronize the larvae metamorphosis, diminishing competitive behaviors (Klaren *et al.*, 2008). This is verified by the results relating to the reduced sole growth heterogeneity in the probiotic group (Table 14) and by the distribution of specimens in the different stages during metamorphosis (Figure 20). This outcome has been reported in *Perca fluviatilis* larvae, using *Bacillus sp*. probiotic in the diet (Mandiki *et al.*, 2011) and in *S.senegalensis* using *S.putrefaciens* pdp11 (Lobo *et al.*, 2014b). Jobling and Wandsvik (1983) established that growth dispersion may be due to a reduced accessibility of food for the less competitive animals. Therefore, the use of Bactocell in the food regimen can diminish the animal handling during the size grading in aquaculture facilities, decreasing the number of stress situations and consequently, increasing growth performance (Lobo *et al.*, 2014a).

In this assay, during the sole weaning, the growth rates diminished in both treatment groups, which could be related to the adaption to inert food. This result have been previously observed in sole (Engrola *et al.*, 2007; Mai *et al.*, 2009).

In different studies of several species it has been stated that this probiotic is transient (*i.e.* do not colonize the gut), so it is necessary to perform a continuous administration during the larvae growth to obtain successful results (Feed Mix, 2005). In relation to the different probiotics (*i.e. Enterococus faecim* and *S.putrefaciens* pdp11) used in Senegalense sole cultivation, the majority of results started to be visually significant after the 40 DAH (Varela

et al., 2010; Lobo *et al.*, 2014a), so it is possible that the administration of probiotics would be also advantageous during and after the weaning phase, where diet is based in inert food (Lobo *et al.*, 2014a). This could be related to a better nutrient administration and handling (Sun *et al.*, 2013). Therefore, for both reasons, it might be necessary to supply a longer pulse of Bactocell probiotic, as it has been done previously with *Pediococcus acidilactici* in trout (Merrifield *et al.*, 2010a), tilapia (Ferguson *et al.*, 2010) or salmon (Feed Mix, 2005) and with *S.putrefaciens* pdp11, improving growth of Senegalense sole (Lobo *et al.*, 2014b). The effect on fish performance could be possible due to the enhancement of digestive activity (vitamins, enzymes and other factors) and immune response (*i.e.* macrophage activity) (Tapia-Paniagua *et al.*, 2012). Additionally, *Enterococus faecim* and *S.putrefaciens* pdp11 administration via live feed in *Solea senegalensis* larvae improved growth, welfare and feed utilization (Sáenz de Rodrigáñez *et al.*, 2009; Lobo *et al.*, 2014a), so it is possible that Bactocell administered via feed may also improve other factors in sole larvae performance, such as feed utilization, digestive enzymes, immune system, among others. Makridis *et al.* (2008) and Avella *et al.* (2011) shown that *Enterococus faecim* and *Shewanella spp.* inhibit *in vitro* the *V. anguillarum* and *Photobacterium damsel subsp. Piscicida*.

The use of lactic acid bacteria in fish has improved the survival rates (Gatesoupe, 1999; Ghosh *et al.*, 2007; García de la Banda *et al.*, 2012). Thereby, according to this data, Bactocell may improve the general fish welfare.

Furthermore, the fish intestinal microbiota plays an important role as a defensive mechanism against pathogens (Ringø *et al.*, 2014). Therefore, is important to understand the effects of this microorganism's and their interaction with the host. The ability to modulate fish intestinal microbiota has been reported for certain microorganisms, such as LAB (Lobo *et al.*, 2014a). It is known that early feeding with supplements like probiotics may modify the gut microbiota and have several effects on larval physiology and morphology (Abelli *et al.*, 2009). PCR-Denaturing Gradient Gel Electrectrophoresis (PCR-DGGE) technique has been used to evaluate the composition of the microbiota and their variation over time (Uchii *et al.*, 2006; Brunvold *et al.*, 2007; He *et al.*, 2009; Merrifield *et al.*, 2010b). This method analyses 16S rDNA, allowing to see the different species present in the intestinal microbiota over the period of study (Tapia-Paniagua *et al.*, 2012). The results obtained in this study from the analysis of DGGE patterns of fish fed with both dietary regimes, may indicate that this probiotic has potential to modulate the gut microbiota of sole larvae and fry. This modulation has been similar to that reported by García de la Banda *et al.* (2012) and Lobo *et al.* (2014a), when they reported that the use of *S. putrefaciens* in the sole diet were capable of modulate the sole intestinal microbiota. Microbiota modulation by oral probiotic bacteria has also been reported in *Scophthalmus maximus (*Ringø and Birkbeck, 1999*)*,

Oncorhynchus mykiss (Kim and Austin, 2006) and *Epinephelus coioides* (Sun *et al.*, 2013). This gut colonization ability in the first larvae stages of *Solea senegalensis* (20 DAH), when the digestive tract is not fully developed (Padrós *et al.*, 2011) might be relevant in relation to the immune system resistance and nutrition capacity, as has been previously described for different probiotic strain administered to farmed fish (Tinh *et al.*, 2008; Sun *et al.*, 2013). Moreover, this modulation may avoid the establishment of opportunistic pathogenic bacteria (Yang *et al.*, 2012).

In summary, notwithstanding the absence of benefits relating growth performance, the use of *Pediococcus acidilactici* may have some potential applications in *Solea senagalensis* as it happens in trout, salmon and shrimp, such as in the immune system, digestion, pigmentation, skeletal deformities and digestive system. As mentioned before, the use of *Pediococcus acidilactici* have increased the number of well-conformed fish in trout (Aubin *et al.*, 2005), salmon (Feed Mix, 2005) and seabass (EFSA, 2009). This product has been used to improve the quality of the animal product, increasing the number of well-conformed fish and reducing the incidence of bone deformities (EFSA, 2009). So, it is necessary to study the effect of Bactocell in the sole skeletal deformities.

Future studies are necessary to optimize dosage rates, administrations forms and timing of feeding in *Solea senegalensis*. More studies are needed to evaluate the effects on the immune response, gastrointestinal tract, nutrition, disease resistance and flesh quality, in order to define if Bactocell is a suitable probiotic candidate for *Solea senegalensis* applications. Regarding the immune system, it would be interesting to test the effect of the stated probiotics (*Pediococcus acidilactici* and *Shewanella spp)* in the respiratory burst activity of sole leukocytes, the stimulation of this bacteria in the innate and adaptive immune system (phagocytes, antibodies, among other components), as was done with *S. putrefaciens* pdp11 in sole (Díaz-Rosales *et al.*, 2009; Tapia-Paniagua *et al.*, 2012). If the probiotic improves the immune system, it is expected that this strain might reduce the incidence of infections (Tapia-Paniagua *et al.*, 2012). It is important to study the dynamics between this probiotic and the pathogens commonly associated with sole to understand if Bactocell is able to inhibit the pathogenic agent. Respectively to the gastrointestinal tract, as shown with the use *S. putrefaciens* pdp11 in the dietary of sole (García de la Banda *et al.*, 2012), it is necessary to understand if *Pediococcus acidilactici* is also capable of reducing the high number of large lipid inclusions inside of enterocytes.

In terms of nutrition, it is relevant to know how the microbial modulation made by Bactocell may enhance the nutrition of the host. As such, it will be possible to deduce the effect of this bacteria in the digestive process, feed digestibility and nutritive utilization as it was done previously (Lin *et al.*, 2004; Burr *et al.*, 2005; Wang *et al.*, 2008). Other possible study is to evaluate if the use of probiotics could enhance the quality of the muscle, as it

was suggested by some authors (Sáenz de Rodrigáñez *et al.*, 2009; Lobo *et al.*, 2014a). For example, to correlate the administration of probiotic to the stress tolerance and resistance, stress response studies can be realized (Varela *et al.*, 2010).

Additionally, the use of marine authochthonous probiotics such as *Enterococus faecim* (isolated from the sole intestine) (Avella *et al.*, 2011) and *S. putrefaciens* (isolated from the skin mucus of healthy gilthead seabream (*Sparus aurata*) (Tapia-Paniagua *et al.*, 2012) could have an effective and positive role in the improvement of performance and welfare, diminishing the stress response to captivity conditions.

The administration of sodium alginate as a prebiotic has demonstrated to be effective in the acceleration of stabilization of the microbiota in the sole intestine. It is also known that this element works as an immunoestimulant in marine aquaculture (Tapia-Paniagua *et al.*, 2012), improving the pathogen resistance (García de la Banda *et al.*, 2012).

IV. Conclusions and future perspectives

Safiestela, SA has started production of sole juveniles in 2012 after a considerable investment in the modernization and upscalling of this unit, placing it in the forefront of the aquaculture in Portugal. The complete control of the entire sole production cycle will give a huge economic and competitive advantage to this company. It was an enormous opportunity to be able to do my internship in this company with great future perspectives.

The internship at Safiestela gave us bases and working tools in various areas of aquaculture. It was possible to understand the functioning of the different phases of the sole life cycle: embryonic, larval, juvenile and adult. It was possible to do all the daily routines, since size grading, transportation, cleaning, feeding, among others. It was also possible to understand how all adjuvants for the proper functioning of the sole cycle work, such as control and handling of inert and live food and all the life support equipment that preserves the system functional and healthy (pumps, pipes, tanks, filters, among others). We also learned how to clean and disinfect each area and material.

This cognition will be an asset to our knowledge of methods and works in aquaculture. The fact that this company is a sole hatchery and it is necessary a special and meticulous care with the sole larvae, gave us a technical expertise in this specific area.

Concerning the assays realized in this internship, the overall results suggested that the commercial probiotic Bactocell did not influence the total length and dry weight of the Solea Senegalense larvae in both assays, with the exception of the 7, 9 and 31th DAH of the second assay ($p<0.05$), where the larvae group supplemented with *Pediococcus acidilactici* bacterial strain exhibited significant higher total length than the control group, with no bacteria administered into the feed regime.

Despite the absence of benefits (no significative differences) relating to growth performance, this probiotic has the capability to modulate larval and fry gut microbiota (demonstrated by the DGGE analysis), so these microorganisms may have positive effects in other specific areas as the immune system, digestion, nutrition, welfare, pigmentation, skeletal deformities.

It should be noted that conducting experimental tests under laboratory conditions or in production scale conditions is different because in the laboratory it is easier to control the different abiotic and biotic variables that can affect the test, due to their smaller scale. However, transference of knowledge from the laboratory to the aquaculture facility is of prime importance, as well as their adjustment to the particular conditions of each aquaculture production, to ensure a more stable and productive growth of this industry.

These results may be used industrially, where probiotics such as *Pediococcus acidilactici, Enterococus faecim* and *S. putrefaciens* should be studied with the aim to increase the performance and agility of the sole production cycle.

To understand if the use of probiotic Bactocell has advantages for the growth of *Solea senegalensis*, more studies are required for longer periods of evaluation. These studies are necessary to optimize dosage rates, administration forms and feeding periods in *Solea senegalensis*. It is necessary to do more studies in relation to the immune response, effect on the gastrointestinal tract, nutrition, disease resistance and to do so, it is required immunological, disease challenge and stress response studies; it is also adequate some *in vitro* studies related to the interaction of *Pediococcus acidilactici* with some pathogens agents common in sole.

In case it is established that *Pediococcus acidilactici* bacterial strain positively influence the growth of this sole species in future studies, this probiotic will be an asset for the company, contributing to its success to a faster and more quality growth of the *Solea senegalensis*.

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VI. Appendices

1. Technical sheet of *Solea senegalensis*

The sole is a fish commonly found in the Mediterranean sea and in the Atlantic ocean and is distributed from the Bay of Biscay to Senegal in the south Atlantic, as represented by the red dots in the Figure A 1 (Arjona *et al.*, 2009).

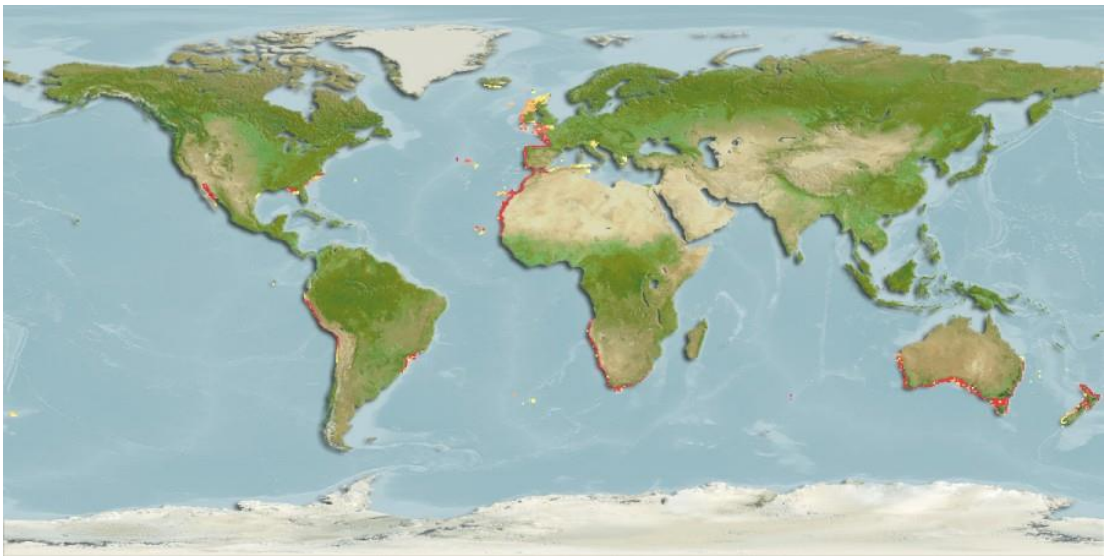

Figure A 1 - Distribution of *S.senegalensis* population worldwide (picture from FishBase (2015)).

This fish species belongs to the *Actinopterygii* class (ray-finned fish), *Pleuronectiformes* order (Flatfishes), *solid* family and *Solea* gender (soles) and presents an oval and asymmetric shape, with both eyes on the right side of the head (fishbases). The dorsal and the anal fin are joined with the tail fin through a membrane. The eye face has a brown-gray color and the blind face presents a clear white coloration (Nelson, 2006). The interradial membrane of the pectoral fin of the eye side is black. This feature distinguishes the *Solea senegalensis* (Figure A 2) from the *Solea solea* which, in turn, has a large black spot on the back side of its fin (FishBase, 2015).

Initially is a pelagic fish, with one eye on each side of its head. During the metamorphosis process, as it grows from the larval to the juvenile stage, one eye migrates to the other side of the body. As an adult, the sole is a benthic fish and their habits and behaviors undergo significant changes (FishBase, 2015).

This is a flat fish that inhabits sandy bottoms or mud with 12 to 100 meters deep, and feeds mainly with polychaetes, amphipods, copepod, isopods and other small crustaceans. Usually, adult soles live in a range of 8.0 to 24.0 °C and, during the winter, they refuge in deep waters, while juveniles are found in most coastal areas, for instance estuarine areas (FishBase, 2015).

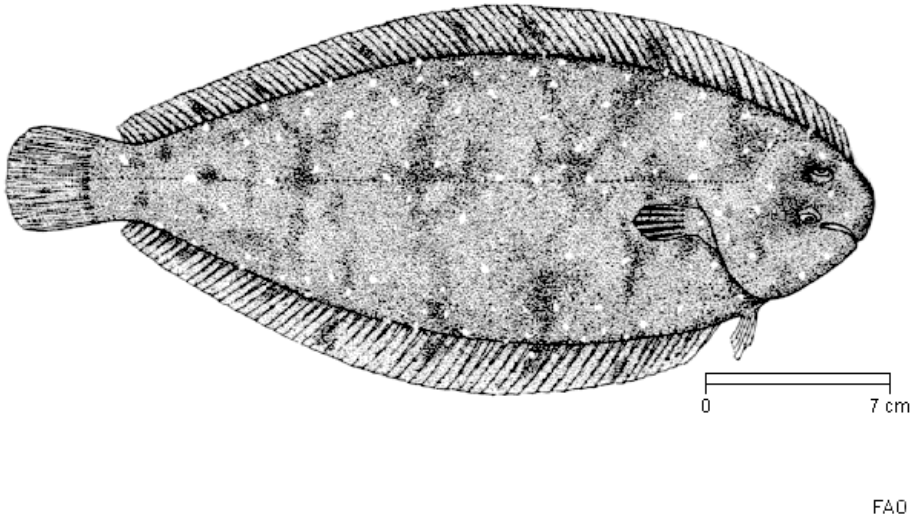

Figure A 2 - *Solea senegalensis* (Picture from FAO (2015)).

Reproduction starts after 3-5 years of age, when they reach 25-30 cm in size. The spawning season occurs annually between February and June (FishBase, 2015) in coastal waters (FAO, 2015). Spawning takes place in shallow coastal waters at temperatures of 6 to 12 °C. The sole females produce, on average, 509 oocytes by kilogram (Dinis *et al.*, 1999) and incubation lasts about 5 days (at 12 °C) and larval phase 35 days (at 18 °C).

The sole is a species produced in extensive onshore tanks and old salt marshes adapted to aquaculture (INE, 2014; DGRM, 2013). It is a species resistant to salinity variations and feed on natural food provided by water changes at high tide (Arjona *et al.*, 2009). Lately, several aquacultures have successively managed to produce this species intensively (Morais *et al.*, 2014a).